THE EFFECT OF CONDENSED TANNIN UPON THE PROTEIN NUTRITIONAL VALUE OF SOLVENT EXTRACTED COTTONSEED MEAL FOR RUMINANT AND MONOGASTRIC ANIMALS

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DECLARATION

The studies presented in this thesis were completed by the author whilst a postgraduate student in the Department of Animal Science, Massey University, Palmerston North, New Zealand. This is all my own work and the views presented are mine alone. Any assistance received is acknowledged in the thesis. All references cited are included in the bibliography.

I certify that the substance of the thesis has not already been submitted for any degree and is not being currently submitted for any other degree. I certify that to the best of my knowledge any help received in preparing this thesis, and all sources used, have been acknowledged in this thesis.

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ABSTRACT

(Feng Yu, 1995 *The Effect of Condensed Tannin upon the Protein Nutritional Value of Solvent Extracted Cottonseed Meal for Ruminant and Monogastric Animals.* Ph D thesis, Department of Animal Science, Massey University, Palmerston North, New Zealand)

A series of indoor experiments were conducted at Massey University and AgResearch Grasslands, Palmerston North, New Zealand, to study the effect of cottonseed condensed tannin (CT) upon the nutritional value of solvent extracted cottonseed meal (CSM) for ruminant and monogastric livestock. Ruminant nutrition experiments were conducted using samples suspended *in situ* in the rumen of fistulated sheep and by incubating samples with rumen fluid *in vitro*, to study effects upon solubility and degradability of cottonseed proteins. Monogastric nutrition experiments were done initially with laboratory rats as a model for production animals such as the pig, and then with pigs. In all cases half of the animals were supplemented with polyethylene glycol (PEG; MW 3500). PEG specifically binds and inactivates CT and can be used to deduce the effects of CT by comparing control animals (CT acting) with PEG supplemented animals (CT inactivated).

1. Experimental varieties of cottonseed and of industrial CSM were analysed for extractable and bound CT and free gossypol, crude protein, oil and fibre. CT was present in the hulls of all varieties, with higher concentrations recorded for high tannin and glandless selections (55 g kg^{-1} and 58 g kg^{-1} DM) than for the multiple host plant resistant and high gossypol selections (38 g kg^{-1} DM). CT was present in trace amounts in the kernels of high tannin selections, but was not detected in the kernels of all other selections. On average for the hulls of all varieties, approximately 22, 60 and 18% of total CT was present in the extractable, protein-bound and fibre-bound forms, respectively. Free gossypol was mainly found in the kernels, with negligible amounts being found in the hulls of the experimental varieties. Kernels of high gossypol selections contained higher concentrations of free gossypol (18 g kg^{-1} DM) than kernels of multiple host plant resistant, high tannin and commercial selections (10-12 g kg^{-1} DM), with free gossypol concentration being very low (0.8 g kg^{-1} DM) in the kernels of glandless cottonseed. A negative correlation ($r = -0.50$, $P<0.05$) between free gossypol in the kernels and total CT in the hulls was found.

The mean crude protein, oil and fibre (neutral detergent fibre; NDF) contents of the kernels were 346, 348 and 228 g kg^{-1} DM, respectively. The hulls contained small

amount of crude protein (37 g kg^{-1} DM) and oil (12 g kg^{-1} DM), and large amount of fibre (NDF; 891 g kg^{-1} DM).

Commercially produced CSM contained 8-15 g CT kg^{-1} DM, due to the presence of some hulls, and 0.8 g free gossypol kg^{-1} DM. The mean contents of crude protein, oil and fibre (NDF) in CSM were 449, 70 and 253 g kg^{-1} DM, respectively. The results are discussed in relation to plant defence mechanisms against insect attack and in relation to the nutritive value of CSM for ruminant and monogastric livestock.

2. The effect of adding cottonseed hulls upon the solubility of protein in unheated solvent extracted cottonseed kernels was studied using both *in vitro* incubation in mineral buffer and the *in situ* polyester bag technique. The latter technique was also used to study effects on rumen DM digestion. Cottonseed hulls contained 51 g CT kg^{-1} DM, with 56 and 20% of the total CT being bound to protein and fibre, respectively; no CT was detected in kernel.

   In the absence of hulls, 42% of the total nitrogen (N) in cottonseed kernel was soluble in mineral buffer *in vitro*, whilst potential *in situ* N solubility and predicted rumen N solubility (corrected for rumen outflow rate) were 99 and 86% respectively. Addition of hulls linearly reduced both *in vitro* N solubility and potential *in situ* N solubility, with 100% hulls addition (i.e. 1 g kernel + 1 g hulls) reducing potential N solubility and predicted rumen N solubility to 94 and 79% respectively. PEG addition had no effect upon the protein solubility of kernels, but increased N solubility in mixtures of hulls and kernels *in vitro* but not *in situ*. Two mg PEG mg^{-1} total CT was shown to reverse the effect of CT in reducing *in vitro* protein solubility.

   Potential *in situ* DM digestion and predicted rumen DM digestion (corrected for rumen outflow rate) were substantially lower for cottonseed hulls (41 and 33%) than for kernels (99 and 88%). Increasing the addition of hulls to kernels lowered the rumen DM digestion of mixtures in a quadratic manner, with increasing rate of hulls causing progressively smaller depressions. Addition of PEG had no effect upon the digestion of kernel DM, but increased potential DM digestibility and predicted rumen DM digestion of hulls to 47 and 40% respectively.

   It was concluded that the high protein solubility of unheated solvent extracted cottonseed kernels can be linearly reduced by the addition of cottonseed hulls, with the magnitude of the reduction being small, and that the presence of bound CT in hulls substantially depressed fibre digestion by rumen micro-organisms.

3. A 24 h *in vitro* rumen incubation procedure was developed to measure the effect of adding cottonseed hulls upon degradation of the 52 and 48 kDa major seed storage proteins present in unheated solvent extracted cottonseed kernels. Proteins were

fractionated using sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the protein bands quantified using imaging densitometry. A set of *in vitro* experiments was conducted, in which degradation rate and potential degradability were measured.

In the absence of hulls, potential degradability of both kernel proteins was very high (99%), with approximately 97% of this taking place within 8 h, and the addition of PEG did not effect degradation. Increasing rates of hull addition reduced the potential degradability of both proteins in a linear manner, but did not affect degradation rate. Equal weights of hulls and kernel (i.e. 100% hulls) reduced potential degradability of the 52 and 48 kDa proteins by approximately 10% units. Addition of PEG increased degradation of both proteins in incubations involving mixtures of hulls and kernels, with 2 mg PEG mg^{-1} CT being required to maximise this effect. However, the increase obtained accounted for only 50% of the depression in protein degradation caused by the addition of hulls. In all experiments, the 52 and 48 kDa proteins were similarly affected by the treatments applied.

It was concluded that *in vitro* rumen degradability of the 52 and 48 kDa storage kernel proteins was very high and close to 100%, and that this could be reduced by the addition of hulls in a linear relationship, with approximately half of the depression in potential degradability caused by hulls being due to effects of CT.

4. The effect of CT in cottonseed hulls on endogenous ileal amino acid flow in the growing rat was evaluated. Twenty-four rats were allocated to four semi-synthetic diets, which contained enzymically hydrolysed casein (EHC) as the sole source of dietary N and chromic oxide as an indigestible marker. Two of the diets contained no hulls while the remaining two contained 50 g kg^{-1} hulls. At each level of hull inclusion, PEG was added to one of the diets. The rats were given their respective experimental diets *ad libitum* for 14 days. Samples of digesta were collected at slaughter from the terminal 15 cm of ileum. The digesta samples were centrifuged and the supernate ultrafiltered. The precipitate plus retentate (MW> 10,000) fraction affords an estimate of endogenous loss.

Inclusion of hulls in the EHC based diet increased ileal flow of total N (1387 vs. 1623 mg kg^{-1} dry matter intake; $p<0.05$), increased ileal flow of total amino acids (23%; $p<0.01$), and significantly increased ileal flow of several individual amino acids. There was no significant effect of PEG and no PEG × diet interaction, showing that the hull effects could not be explained by action of CT. The presence of some hulls in commercial CSM will contribute to lowering apparent ileal amino acid digestibility, due to its effect in increasing endogenous loss.

5. The effect of adding cottonseed hulls to casein and to cottonseed kernel based diets on the apparent and true ileal digestibility of N and amino acids, and the proportion of this effect accounted for by CT, was determined using the growing rat. Sixty rats were allocated randomly to 10 semi-synthetic diets, containing either casein (4 diets) or purified unheated solvent-extracted cottonseed kernel (6 diets) as the sole protein sources, with chromic oxide added as an indigestible marker. Two of the casein diets contained no hulls whilst the remaining two diets contained 70 g hulls kg^{-1}. Two of the cottonseed kernel based diets contained no hulls, with two containing 23 g hulls kg^{-1} and the remaining two containing 46 g hulls kg^{-1}. For each pair of diets, PEG was either included or excluded. Samples of digesta were collected at death from the terminal 15 cm of ileum.

   The inclusion of hulls depressed the apparent and true ileal digestibility of N and amino acids, but with the response differing between diets. With the casein based diet the mean apparent and true ileal amino acid digestibilities were significantly depressed from 0.89 and 0.96 to 0.85 and 0.92, respectively, by the inclusion of hulls in the diet, and addition of PEG then restored these to 0.89 and 0.95. All of the depression could be explained by the CT content of the hulls. However, with the cottonseed kernel based diet the response fell into three categories. The apparent and true ileal digestibilities of the essential amino acids cystine and methionine were not affected by hull addition, ileal digestibilities of leucine, isoleucine, lysine, threonine and valine were markedly depressed by hull addition with approximately 50% of the depression being explained by CT, whilst the ileal digestibilities of histidine, arginine and phenylalanine were depressed by hull addition but little or none of this effect could be explained by CT. With the cottonseed kernel based diet it seems that unknown components of the hulls other than CT also depressed the apparent and true ileal digestibility of N and amino acids.

6. The effects of heat treatment, with or without the addition of cottonseed hulls, on the chemical composition of CSM and upon reactivity of the CT were studied. Heat treatment (100°C for 2 h, in a forced draught oven) reduced the concentrations of free gossypol and fluorodinitrobenzene (FDNB) available lysine by small amounts, reduced measurable total CT content by 13%, reduced the solubility of total N, and reduced potential degradability of the 52 and 48 kDa cottonseed storage proteins by mixed rumen micro-organisms. Addition of hulls further depressed solubility of total N and ruminal degradation of the two major storage proteins in cottonseed kernel. The action of PEG *in vitro* indicated that only part of the depression caused by hull addition could be explained by the presence of CT in the hulls, and that the effects of CT upon N solubility and potential degradability in heated CSM were similar to that

in unheated CSM. Addition of hulls also substantially reduced FDNB available lysine.

Although application of heat inactivated 13% of the total CT such that it could no longer be detected with butanol/HCl, it did not seem to change the reversible reactivity of CT with kernel proteins. Commercial CSM produced from the Brisbane mill had a lower total CT content, lower N solubility and ruminal protein degradation rate than CSM produced from the Narrabri mill, but a similar level of FDNB available lysine.

7. The effect of CT from heated and unheated cottonseed on the apparent ileal digestibility of amino acids for the growing rat and pig was determined. In Experiment 1, twenty-four rats were allocated to four semi-synthetic diets, which contained cottonseed kernel/hulls as the sole protein source. Two of the diets contained unheated solvent extracted cottonseed kernel/hulls while the remaining two diets contained similar material but which had been heat-treated by autoclaving at 110°C for 120 min. In Experiment 2, twelve rats and twelve pigs were fed four semi-synthetic diets containing commercial CSM as the sole protein source. Chromic oxide was added to all diets as an indigestible marker. For each pair of diets in both experiments, PEG was either included or excluded. Ileal contents from the terminal 15 and 45 cm of ileum were collected at slaughter for the rats and pigs, respectively.

Apparent ileal amino acid digestibility for rats fed the cottonseed kernel/hulls diet was significantly depressed by the heat treatment, particularly for lysine and threonine. On average, apparent ileal amino acid digestibility in the diets without PEG was decreased from 0.80 to 0.70 by heat treatment. Dietary cottonseed CT depressed apparent ileal protein digestibility in the pig and in the rat. The addition of PEG to the diets significantly increased the apparent ileal digestibility of N and some amino acids for the pigs and the rats. The mean increase in apparent ileal digestibility due to PEG addition for the 14 amino acids was 2% units in both species fed the commercial CSM diets, and 2 or 4% units in rats fed the unheated or the heated cottonseed kernel/hull diets, respectively. The effect of PEG was similar in the heated and unheated cottonseed kernel/hulls for most amino acids, but responses to PEG for lysine, threonine and tyrosine were greater in heated than unheated CSM.

For several of the amino acids there were significant animal species differences in apparent ileal digestibility. Studies into the effects of cottonseed CT should be carried out in the target animal species. The commercial CSM had a low apparent ileal amino acid digestibility overall, particularly for the essential amino acids lysine and threonine. It was concluded that effects of heating did not eliminate the reversible reactivity of

cottonseed CT on amino acid digestion in rats and pigs but rather appeared to increase it for threonine, tyrosine and lysine in Experiment 1, causing large reductions in apparent ileal digestibility.

It was concluded that CT in CSM are mainly in the protein- and fibre-bound forms (80%), with only 10-20% of CT being extractable. In the absence of heat, adding graded levels of hulls reduced both the solubility and degradability of protein by rumen micro-organisms, giving a rumen ' by-pass' effect. However, the magnitude of these effects was small and they are not regarded as being nutritionally significant under practical conditions of ruminant nutrition. Adding hulls significantly depressed the digestion of amino acids in the small intestine of the monogastric animals. This effect is considered large, and is probably one of the factors responsible for the generally low levels of amino acid availability found for commercially produced CSM. Approximately half of the 'hull effect' is due to their content of CT and the cause of the other 50% is unknown. As there is no CT in cottonseed kernels, this explains the lack of response to PEG when the diet does not contain cottonseed hulls. The study has also shown that the large amount of heat applied during normal commercial CSM processing reduces the digestion of amino acids by monogastric animals. A combination of heat and CT from hulls is especially damaging for lowing ileal amino acid digestion in monogastric species, particularly for the limiting essential amino acids lysine and threonine.

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GENERAL INTRODUCTION

The English word "cotton" comes from the Arabic word *qutun* or *kutun*. The same root is also found in many other languages: *katoen* (Dutch), *coton* (French), *cottone* (Italian) and *algodón* (Spanish). The scientific term *Gossypium* is derived from the Arab, Persian and Afghan words *goz*, *gozah* and *gozeh*, respectively (Müller 1958).

The time when cotton fibre was first utilised by man is not known. The oldest archaeological record of cotton textiles, which dates back to about 3,000 B.C., was found in the valley of the Indus river in West Pakistan (Gulati and Turner 1929). The first reference in the literature to cotton, so far as is known at present, is to be found in a Hindu Gig-Veda hymn (Scherer 1916). This hymn was written about 15 centuries B.C. and mentioned that cotton was being used for weaving in India at that time. Nearchus reported about 327 B.C. on cotton growing in the Indus river valley and around the shores of the Arabian and Persian Gulfs (Handy 1896).

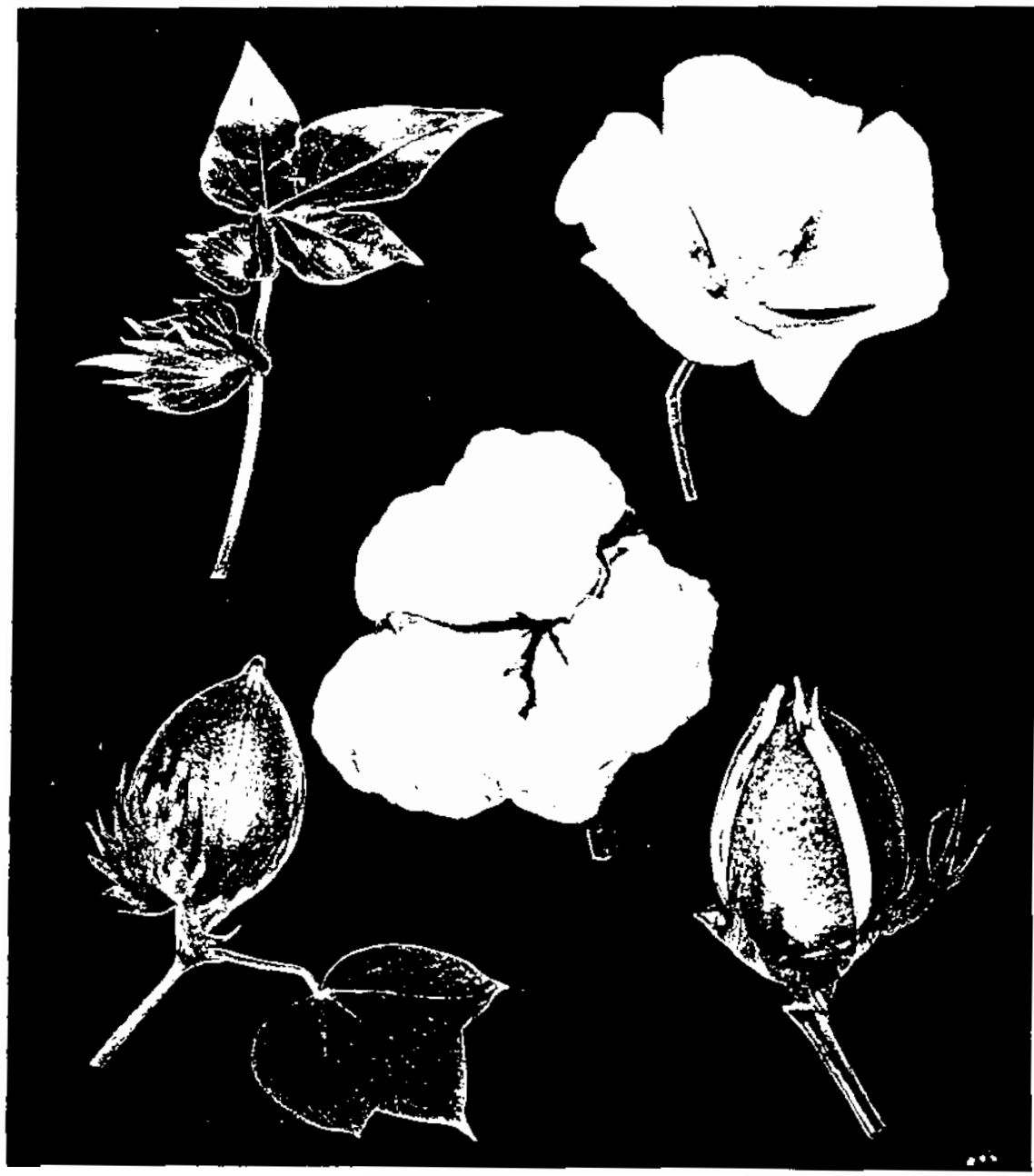
Cotton plants are annual shrubs usually cultivated in almost every tropical country, as well as in many sub-tropical parts of the world (Plate 1). Harland (1936) showed that only four species embraced the whole vast diversity of the cultivated cottons. Two species, *Gossypium herbaceum* and *Gossypium arboreum*, have n=13 chromosomes, and two species, *Gossypium hirsutum* and *Gossypium barbadense*, have n=26 chromosomes. The great bulk of the cotton grown commercially throughout the world is of *Gossypium hirsutum* genotypes, whilst *Gossypium barbadense* genotypes are grown to a much smaller extent (Berger 1969).

The fruit of the cotton plant, known as the boll, is a spherical or ovoid capsule varying in form and size with each species. The young boll (ovary) develops rapidly and reaches full size about 21 days after pollination. An additional 20 to 50 days elapse before the boll is mature and ready to open. The time from flowering to boll opening ranges from 40 to 70 days, varying to some extent with the variety but primarily with temperature, rainfall, sunlight and soil fertility. Development of a cotton boll is shown in Plate 2. The boll contains the seed, lint (long, thickened, white or creamy coloured fibres) and fuzz (very short, usually white but sometimes coloured fibres strongly attached to the seed coat).

The seed is an ovoid, more or less pointed, dark brown structure and ranges in length among species from 6-12 mm; the weight fluctuates between 16-17 g per 100 seeds. Cotton seed is a by-product of producing cotton, with the main product being lint (i.e. cotton fibre). The ratio of seed weight to lint weight is roughly 1.7 to 1 (Frank 1987). Seed is separated from lint by passage through a gin. The lint, pressed and

Pla. 1 Irrigated cotton field in Oklahoma (U.S.A.)

Pla. 2 Development of a cotton boll. 1) Square or young flower; 2) Open flower; 3) Full-size unopened boll; 4) Boll in process of opening; 5) Fully opened boll ready for harvest



packaged in bales for convenience in handling, is shipped to a textile mill for processing into yarn and fabric. The seed, except for a small proportion set aside for planting and other uses, is transported to a crushing mill for processing into cottonseed oil and cottonseed meal (CSM). Oil is the most valuable product from cottonseed and is consumed almost entirely as food. The second most valuable product of cottonseed is CSM, which is used for livestock feed (as a protein supplement). CSM contains no less than 360 g protein kg^{-1}, which should have relatively good nutritional quality. However, its nutritional value is lower than that predicted on the basis of its chemical composition. This is thought to be due to the effects of processing of cottonseed during oil extraction, and to the presence of several anti-nutritional factors, such as gossypol (Frank 1987) and condensed tannins (Terrill *et al.* 1992).

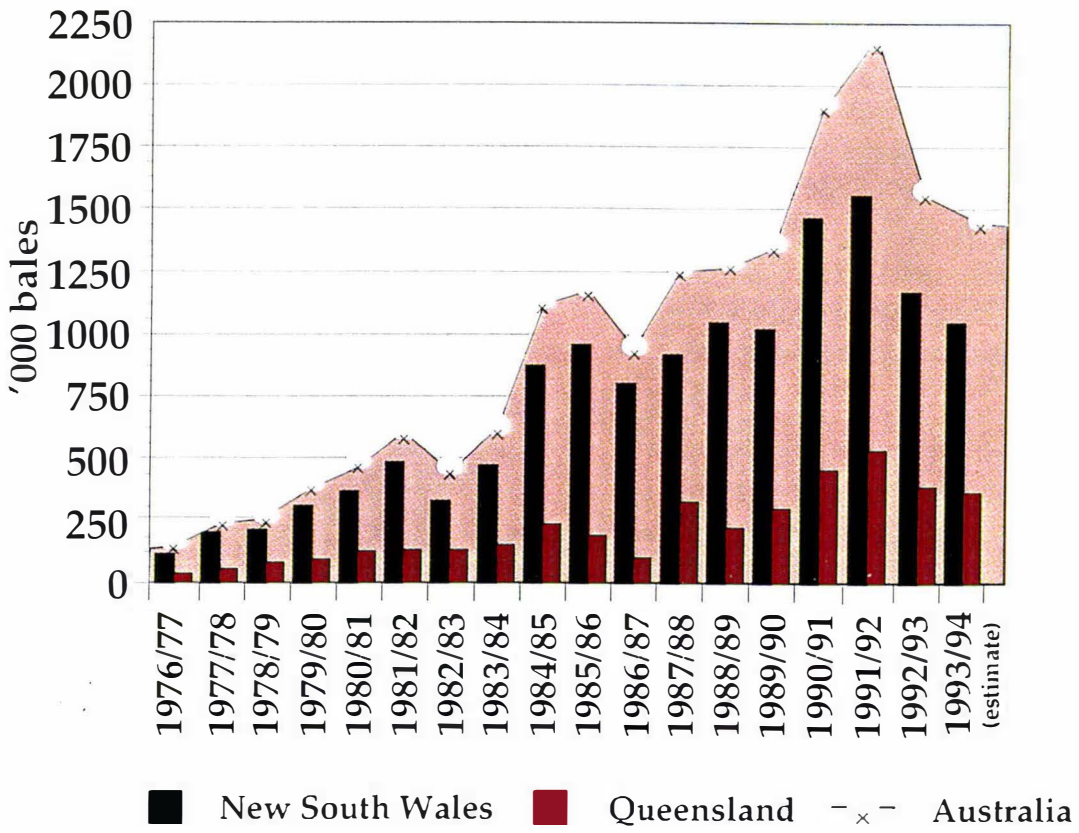


Figure 1 Australian cotton production (Source: The Australian Cottongrower's 1993 Cotton Year Book & CRDC calculations)

In both economic and social terms the cotton industry is a vital part of Australian life. Cotton is Australia's fifth largest rural export earner. Over 92% of the cotton crop is exported, annually netting the nation around $A800 million in offshore sales (CRDC 1994). Despite its considerable economic impact, Australia's modern cotton industry is still relatively young. It emerged in western New South Wales and Queensland in the early 1960s and has experienced its most significant growth during the past decade (Figure 1). In last season (1993-1994), the area planted to cotton in Australia was 262,000 ha, and total harvest was 1.4 million bales (CRDC 1994). Generally, cotton fibre produced in Australia has an excellent reputation both within Australia and overseas for its quality and character. However, the nutritional quality of CSM produced in Australia for ruminant and monogastric livestock is unknown well. Therefore, this thesis investigated the protein nutritional value of meals derived from Australian cottonseed for ruminant and monogastric livestock. The research conducted in this thesis was fully funded by Australian Cotton Research & Development Corporation (CRDC).

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Chapter 1

THE NUTRITIVE VALUE AND UTILISATION OF COTTONSEED MEAL FOR RUMINANT AND MONOGASTRIC ANIMALS: A REVIEW

1. 1 INTRODUCTION

The ever increasing cost and scarcity of conventional feedstuffs in the world, particularly in the developing countries, has led to the search for alternative animal feedstuffs which are not consumed directly by humans. One of the major potential protein sources is oilseed products.

High growth rates for consumption of oilseed meals will probably occur especially where meat production is rising, such as in the U.S.A., South America, Southeast Asia and China. Over the longer term, consumption will also increase in Central Europe and the former Soviet Union, but should stabilise in the European Union (EU), now the world's biggest user (Rabobank 1995). In 1993, the EU accounted for 29% of world oil meal consumption, followed by the U.S. (18%), China (9%), India (6%) and Japan (5%). Between 1985 and 1993, world consumption increased by 3%, to 146.7 million metric tons (mmt). Of the 12 major oil meals produced, soya bean meal was the most consumed, at 76.0 mmt in 1993. Cottonseed meal (CSM) ranked second, at 14.6 mmt, followed by rapeseed meal, corn gluten meal, sunflower meal, fish meal, groundnut meal, corn germ meal, palm kernel meal, copra meal, linseed meal and sesame seed meal (Oil World Annual 1993). In the cotton industry, production of lint (i.e. cotton fibre) gives the largest financial return, followed by sale of oil extracted from the seed. CSM is the residue after oil extraction from the seeds, and is regarded as a by-product. CSM is potentially a very important protein source for livestock production, particularly in the cotton producing areas. Therefore, further nutritional investigation of CSM needs to be done, since it is cheaper and more readily available than other conventional protein concentrate feeds, such as soya bean meal or fish meal.

Although CSM is commonly used as a protein supplement for ruminant animals, its use for human and monogastric farm animals has been limited. This is attributable to its high fibre content and its content of naturally occurring anti-nutritional factors (ANFs), such as gossypol (Frank 1987; Martin 1990), tannins (Balogun *et al.* 1990; Terrill *et al.* 1992a), cyclopropenoid fatty acids (Phelps 1966; Waldroup 1981) and its lower level of lysine compared with soya bean meal (Yoo and Hsueh 1985; Batterham *et al.* 1990).

In this overview the following topics will be discussed; the growth of cotton and products from cotton, the characteristics of cottonseed products, the ANFs in CSM, commercial seed processing methods, the effect of heat and solvent extraction upon the nutritive value of cottonseed products, efficiency of CSM for animal production, the feeding value of whole cottonseed and its hulls for ruminants and some future prospects.

1. 2 THE GROWTH OF COTTON AND PRODUCTS FROM COTTON

1. 2. 1 The Growth of Cotton

Cotton is the most important of the plant fibres and is used for a variety of purposes, but especially in the manufacture of clothing for mankind. The fibres are produced by the seed coat of various species of *Gossypium*, the most economically important member of the family ***Malvaceae***, and when separated from the seed the fibre is known as "lint".

Gossypium is a large and very variable genus including numerous wild and several cultivated species, showing a wide range of morphological types and distinct genotypic differences. The genus *Gossypium* was described first by Linnaeus (Berger 1969), whilst Watt (1907) described all that was then known of the botany of the cotton plant and much of its agricultural history. Modern work on the crop began with Harland's (1936) analysis of the nature of the species distinctions among the cultivated cottons.

The growth of the cotton plant is greatly influenced by its environment. The cotton plant ripens its fruits in succession over a period of weeks or even months, in contrast to plants like maize, wheat or potatoes which ripen their produce virtually in one short flush. This characteristic of the cotton plant has an important bearing on the management of the crop. Cotton requires a long, warm growing season, and consequently the cultivation of cotton is confined to the warmer latitudes. At any stage of its development the cotton plant is very sensitive to frost. Cotton is a long-season plant, requiring a minimum of 180 to 200 frost-free days. In addition, cotton requires from four to five months of uniformly high temperatures during the growing season. For optimum growth, an average growing season temperature of 21 to 22°C is needed. Sunshine is of great importance to proper development of the cotton plant, particularly during the periods of early growth and full bloom. Rainfall during the growing season is also very important. To provide adequate soil moisture for the crop, a minimum of 500 mm of rainfall is required annually, with 175 to 200 mm being required to be well distributed over the growing season. Cotton is grown on a large variety of soils. Sandy loams, loams and well-granulated clay loams are considered best (Berger 1969). In the Australian cotton industry, irrigated cotton has been the major component in the past few decades, but the limited availability of water constrains farm production in all years, because water demand for cotton production always exceeds supply (Cull and Robson 1994). Rain-grown cotton has become an increasingly important component of the Australian industry over the past ten years. Given good prices and rainfall, the area of the crop grown with rain will exceed 50,000 ha. It is also important to note that during

droughts, irrigators are forced to produce semi-raingrown cotton (Constable *et al.* 1994).

Two grades of cottonseed are commonly found. These are the glanded cottonseed and the glandless cottonseed. The traditional varieties of glanded cottonseed contain gossypol. The glandless cottonseed is virtually gossypol-free. An evaluation of glanded and glandless cottonseed grown in Texas is summarised in Table 1. 1. Mean assay values were generally similar for the whole seed, kernels and solvent-extracted meal for the glanded and glandless varieties, except that the glandless cottonseed kernels averaged 2% more oil content than the glanded kernels. Amino acid profiles were similar for the glanded and glandless seeds. Both typesof cottonseed contained an amount of 40 g available lysine kg^{-1} DM (Lawhon *et al.* 1977). However, the glandless cotton has not been grown much, because of its increased susceptibility to insect attack (Lusas and Jividen 1987).

Table 1. 1 Mean (eight varieties) chemical compositions (g kg^{-1} DM) of glanded and glandless cottonseed and their products (Source: Lawhon *et al.* 1977)

Products and assay	Glanded cottonseed	Glandless cottonseed
Whole cottonseed		
oil	210	211
protein (N × 6.25)	231	225
kernels in seed	617	596
Cottonseed kernels		
oil	378	397
protein (N × 6.25)	393	389
crude fibre	16	17
total gossypol	12	0.2
Hexane-extracted meal		
oil	8	8
protein (N × 6.25)	632	626
crude fibre	27	28
total gossypol	16	0.2

1. 2. 2 Uses of Cotton Plant Products

Cotton comprises approximately half of all domestic textile used. It possesses a unique combination of basic qualities, which accounts for it being the leading fibre in terms of quantities consumed. The washability of cotton has proved one of its outstanding qualities. Cotton is 25% stronger wet than dry, and no other fibre surpasses it in ability to withstand heat during home laundering and drying. Cotton's excellent resistance to rubbing is also a major consideration where serviceability of outer garments is needed. It is a very strong fibre, which is a quality of primary importance in many textile uses. Cotton is moreover an absorbent fibre, which is highly desirable for apparel as well as for

other textile uses. Another aspect of cotton's versatility lies in its adaptability for use in textiles suitable for warm weather as well as cold weather wear. Clothing uses vary from sheer, soft, crisp materials to heavy, coarse, strong fabrics. In the past decade the uses of cotton have been extended through chemical modification, and finishing treatments have been developed which improve the appearance of the final product and impart easy-care properties.

Low-grade fibre, waste products and fuzz are consumed in the production of felts or bats used in mattresses and other bedding products and in upholstery for furniture. Lower quality cotton is used as a raw material in the manufacture of high grade writing paper and rayon, and in the chemical industry, for making photographic and X-ray films and for the production of explosives.

For nearly three-quarters of a century after the saw-ginning machine was invented, cotton seed was regarded as a waste and a nuisance. For purposes of processing into useable items, cotton seed is divided into five products: 1) cotton seed oil, 2) cake and meal, 3) cotton seed hulls, 4) linters and 5) waste (Frank 1987). The proportions of the five primary products of the cottonseed crushing mill are shown in Table 1. 2. The oil is the most valuable of the five products, accounting for 50 to 55% of the total value of seed products (Berger 1969). Cottonseed oil is one of the most important of the world's semi-drying oils and is primarily a food product, used in cooking and salad oil, mayonnaise, margarine and many other products. The second most valuable product of cottonseed is cake or meal, which is used for livestock feed (as a protein supplement). Hulls are the least valuable of the four main products obtained from cottonseed. Like meal, they are used almost entirely in the feeding of livestock, but as a carbohydrate roughage, and to some extent in making synthetic rubber, lubricating oils and certain types of plastics. Linters have a wide variety of uses, including making rayon, explosives, film, shatter-proof glass and plastics.

Table 1. 2 Product distribution in the cottonseed crushing industry (Source: Williamson 1983)

Products	% of initial weight
Crude oil	16.0
Meal	46.1
Hulls	25.8
Linters	8.0
Waste	4.1

Another by-product from the cotton plant is cotton gin trash. Brown *et al.* (1979) defined cotton gin trash as a waste product of the cotton industry that is a mixture of stems, leaves, cotton lint, and a few cottonseeds that have escaped the ginning process. Cotton gin trash can be used in fattening rations for ruminant animals, but has a value of only 70% that of cottonseed hulls (Jones *et al.* 1957).

1. 3 COMMERCIAL PROCESSING METHODS OF COTTONSEED MEAL

Commercial processing of cottonseed is generally carried out by one of four basic methods: hydraulic pressing, screw pressing, prepress solvent extraction, or direct solvent extraction. However, hydraulic pressing is a very old method which is seldom used today (Norris 1982). Before cottonseed is processed, especially by hydraulic and screw pressing, and prepress solvent extraction, the general approach is inactivation using cooking. Oil is then removed from the cooked cottonseed flakes by one of the four above-mentioned methods.

One of the many objectives during processing cottonseed is binding free gossypol pigments in the meal and preventing the pigments from being extruded into the oil. At the same time, damage to the meal protein has to be minimised. Processing conditions influence the content of free gossypol in CSM to a greater extent than they influence the content of total gossypol (Frank 1987).

1. 3. 1 Dehulling and Separation of Hulls

The processing of seed follows similar lines with all processes, although each mill operates under specially selected conditions to obtain maximal efficiency and highest quality products with the seed available. The seed brought to the mills is cleaned and then freed of linters by delintering machines. After removal of the linters, the seed is transferred to the dehulling machines, which are designed to cut or crack the seed so that the hulls are loosened from the kernels. Consequently, the cracked seeds are passed through a series of beaters, shakers and separators that separate the kernels from the loosened hulls. Different proportions of loosened hulls are then removed, depending on the processing methods for oil extraction, and this will be discussed further in Section 1. 3. 7.

1. 3. 2 Cooking of Cottonseed

It is universally recognised that oil seeds yield their oil more readily to mechanical expression after cooking, but a complete explanation of why this is so is lacking. It is

certain that the changes brought about by cooking are complex and that they are both chemical and physio-chemical in nature.

The primary objectives of the cooking process are: a) to coagulate the proteins in the seed causing coalescence of oil droplets and making the seed permeable to the flow of oil and b) to decrease the affinity of the oil for the solid surfaces of the seed so that the best possible yield of oil may be obtained when the seed is subsequently pressed. Another prime purpose of cooking is to bring about destruction or deactivation of free gossypol. Cooking also helps to complete the rupture of the oil cells, increases the fluidity of the oil, insolubilizes the phosphatides and other impurities, prevents crumbling of the meats and destroys moulds and bacteria (Bailey 1948; Norris 1982).

In good cooking practice, flaked cottonseed kernels (about 0.2 mm thick) are brought to approximately 12-15% moisture by the time they are in the top kettle of the cooker, where the temperature is increased rapidly to 88°C or higher, to inactivate the enzyme systems and prevent free fatty acid rise during cooking. Heating should be continued in the presence of not less than 12% moisture until the temperature reaches about 105°C, and then the moisture content is reduced to a value suitable for efficient pressing. Cottonseed kernels are usually kept in the cooker for 30-120 min, and leave the cooker at a temperature of 110-132°C (Norris 1982).

1. 3. 3 Hydraulic Pressing

The term 'hydraulic pressing' is often used in reference to batch pressing in general (Norris 1982). Hydraulic pressing was one of the principal methods for processing cottonseed in 1940's. In 1945 approximately 95% of the United States seed was processed by hydraulic pressing (Harper 1966). In recent years, however, increased mechanisation and higher labour costs have made hydraulic pressing of oil seeds uneconomical in practically all cases. Today, there is no appreciable volume of cottonseeds hydraulic pressed (Norris 1982).

The completeness with which the oil is recovered by mechanical expression is influenced by a number of factors related to the affinity of the oil for solid material in the seed. These include the moisture content, the method of cooking, and the chemical composition of the seed; damaged seed generally retains oil more tenaciously than seed of good quality. The maximal pressure obtained during hydraulic pressing is about 141 kg $(cm^2)^{-1}$. The residual press cake has an oil content of 45-75 g kg^{-1} and a free gossypol content of 0.4-1.0 g kg^{-1} (Berardi and Goldblatt 1980).

1. 3. 4 Screw Pressing

Screw pressing or continuous expellers have now replaced hydraulic presses for the mechanical extraction of cottonseed oil. They are also used extensively throughout the world for the expression of oil from soya bean, flaxseed, peanuts, and almost every other variety of oil seed (Norris 1982). Today, screw press plants are increasingly being used as prepress for solvent extraction systems or are being replaced by direct solvent extraction systems (Norris 1982).

A screw press is essentially a continuous device for gradually increasing the pressure on material fed so it as the latter progresses inside a closed barrel, with provisions for the oil to drain out as it is squeezed from the feedstock. A column or plug of compressed meal is formed at the discharge end of the barrel, acting like a hydraulic presshead with new cake being formed at the end as cake is expelled past a choke device. Fresh feed is forced in by feed augers against the frictional resistance of the plug at the choke, thus creating a hydraulic pressing, equivalent to that of a hydraulic press ram (Tindale and Hill-Hass 1976). Labour required is much less than that necessary for hydraulic pressing, but this is at the expense of higher power requirements and maintenance costs; however, the greater oil yield and reduced labour more than make up for the increased power and maintenance.

In screw pressing, pressures up to about 1408 kg $(cm^2)^{-1}$ are obtained. The oil content of the press cake ranges from about 25 to 50 g kg^{-1} and the free gossypol from 0.2 to 0.5 g kg^{-1} (Berardi and Goldblatt 1980).

1. 3. 5 Prepress Solvent Extraction

The screw press can be used in two ways to expel oil from oilseeds: first, as a high-pressure operation to lower residual oil contents (see Section 1. 3. 4); second, as a pre-press operation prior to solvent extraction (Ward 1976). These two operations are depicted in Figure 1. 1 as simple flow diagrams. In both cases, the four stages which influence pressing efficiency are: a) seed preparation, b) cooking, c) screw pressing, and d) separation of solids from expelled oil and return to cooking/screw press. What is correct in seed preparation and cooking for the high pressure operation is not always suitable for the same seed when prepressing is used. When high pressure pressing, the objective is to obtain the maximum amount of oil and cake leaving the press consistent with capacity and running costs. In a prepress operation, the overall objective remains the same but is based on the output of both press and extractor. Figure 1. 2 shows a detailed flow diagram for the prepress solvent extraction of cottonseed, used by Cargill Oil Seeds at Narrabri, NSW, Australia.

During prepress solvent extraction about two-thirds of the oil is pressed out mechanically, and the press cake is reflaked and extracted with a petroleum hydrocarbon

solvent, generally commercial hexane (see Section 1. 3. 6). After desolventization, the extracted meal contains from 4 to 10 g oil kg^{-1} and 0.2 to 0.7 g free gossypol kg^{-1} (Berardi and Goldblatt 1980).

Most of the mills solvent-extracting cottonseed do so by the prepress route. At first this was probably the response to problems arising in the handling of 'fines' and difficulties in detoxifying the extracted flakes when direct extraction was used. Other advantages of prepressing include the need for only a minimum sized solvent plant, since most of the oil is removed in the prepressing step, and the production of meal of high protein quality. Disadvantages are higher initial equipment costs, and usually higher power requirements and repairs (Norris 1982).

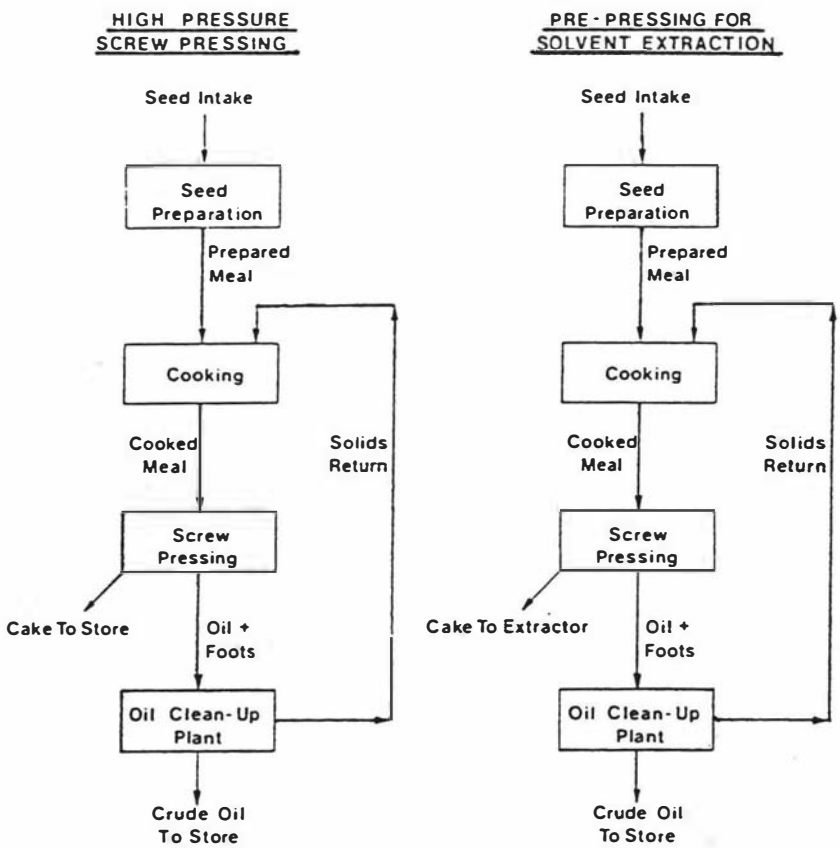


Fig. 1. 1 Flow diagrams for high pressure screw pressing and the prepress solvent extraction of cottonseed (Source: Ward 1976)

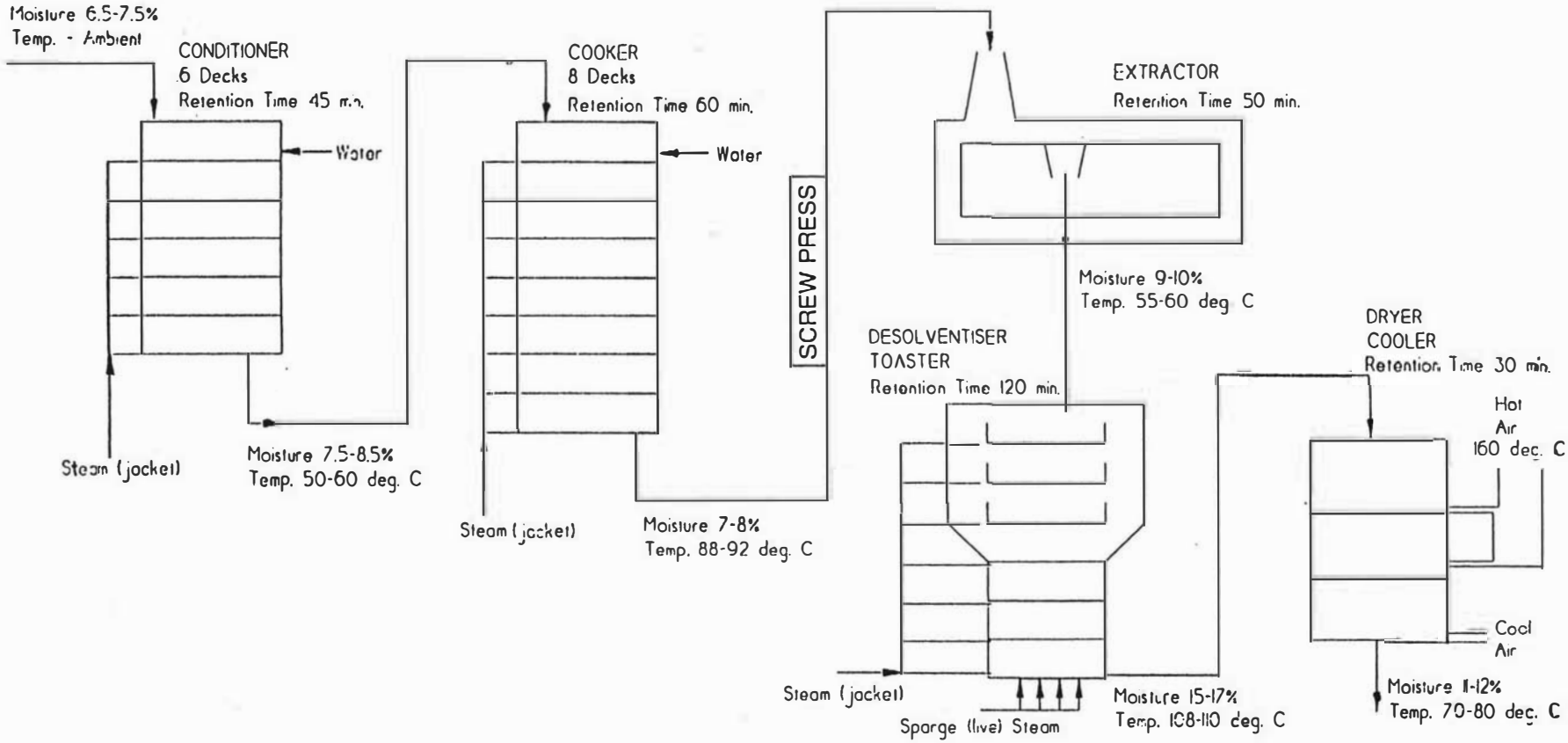


Fig. 1. 2 Detailed flow diagram of the prepress solvent extraction of cottonseed used by Cargill Oil Seeds at Narrabri, NSW, Australia (M. Mittasch, personal communication).

1. 3. 6 Direct Solvent Extraction

Preliminary rupture of the walls of the glands by moistening or grinding, permits rapid extraction of gossypol by any solvent in which the pigment is soluble (Bailey 1948). Gossypol is soluble in polar organic solvents such as ether or acetone but insoluble in hexane or water. Typically, commercial hexane extraction of cottonseed removes a relatively small portion of the gossypol with the oil (Lusas and Jividen 1987).

Alternatively, the cooked cottonseed kernels may be extracted directly with solvent without prepressing. Residual oil in meal obtained by direct solvent extraction (hexane) averages slightly higher than that resulting from prepress solvent extraction but is close to 10 g kg^{-1}. The free gossypol content is also distinctly higher and generally ranges from 1.0 to 5.0 g kg^{-1}. The total gossypol content of the meal obtained is in the range 5-12 g kg^{-1}, depending primarily on the seed used and the conditions used in the preparation of meats prior to extraction of oil (Berardi and Goldblatt 1980). Consequently, the bound gossypol varies. In some direct solvent extraction mills, the kernels are not cooked before extraction (Hron *et al.* 1982; Frank 1987). Since minimum heat treatment is involved, oil produced by solvent extraction is of high quality, and the meal contains protein subjected to minimal damage, due to the effects of heat.

Removal of gossypol by solvents other than the widely used commercial hexane has been investigated extensively. Reviews of removal of gossypol by extraction with polar solvents were published by Berardi and Goldblatt (1980), Hron *et al.* (1982), Norris (1982), Frank (1987) and Lusas and Jividen (1987).

Techniques have also been developed to mill glandless cottonseed in the presence of hexane, then separate the intact, heavier gossypol glands by the liquid cyclone process (Vix *et al.* 1971; Gardner *et al.* 1976). Also, an air classification process has been developed to separate intact gossypol glands from solvent-extracted ground cottonseed flour (Kadan *et al.* 1979; Decossas *et al.* 1982). Although such solvents and techniques have generally been successful in reducing the gossypol content of CSM to quite low levels, oil mills in the world have not yet adopted any of these solvents and techniques because of technical or economic problems or both. Hron *et al.* (1982) has summarised potential biorenewable solvents, the basic processes for each, and the advantages and disadvantages of the proposed solvents compared to the present hexane system.

1. 3. 7 The Role of Hulls in Processing Methods

Wherever practicable, oil seeds are partially dehulled before they are extracted. In practical mill operation, the greatest yield of oil is obtained by finely balancing the degree of separation attained. If an attempt is made to separate all hulls from the kernels, the

hot meats (kernels) can block the extruder during screw pressing and prepress solvent extraction, thus disrupting processing. Therefore, leaving a certain amounts of hulls with the kernels to facilitate passage through the extruder is necessary, prior to solvent extraction of the remaining oil (R. Spencer, personal communication). However, if an excessive amount of hulls is left in the kernels, the crude protein content of CSM will be reduced, and there will be an undue loss of oil from adsorption by the hulls (Norris 1982).

In general, the choice of how much hulls is left with the meats (kernels) depends on what kind of operating system is being used in the mill and what kind of products are being produced. For instance, CSM produced by Cargill Oil Seeds, Narrabri, Australia contains lower crude protein levels (370 g kg^{-1}) and a higher proportion of hulls comparing with CSM produced by Cargill Oil Seeds, Brisbane, Australia (410 g protein kg^{-1}). The processors may use many modifications of the general techniques for their purposes.

1. 4 CHARACTERISTICS OF COTTONSEED PRODUCTS

The value of cottonseed products can vary quite extensively depending on the cultivar, region, growing conditions and processing methods. About 50% of the CSM consists of crude fibre plus nitrogen-free extract fractions. There are relatively small quantities of monosaccharides and disaccharides and only trace amounts of starch (Jones 1981). Some measures of the composition of cottonseed products relative to other oilseed meals are given in Table 1. 3.

1. 4. 1 Protein, Fibre, Oil and Minerals

Cottonseed meal is commonly adjusted to a defined protein content at the mill by varying the completeness with which the hulls are separated from the kernels or by adding ground hulls to the meal (Swern 1982). To be classed as prime quality, CSM should contain no less than 360 g crude protein kg^{-1}. The three commercial grades are 360 g kg^{-1}, 410 g kg^{-1} and 430 g kg^{-1}, 410 g kg^{-1} being the most common (Anon 1985). However, the undehulled CSM, sometimes referred to as undelinted CSM, has a protein content of 260-300 g kg^{-1} (FAO 1975). For statistical purposes, the crude protein is taken to be 410 g kg^{-1}, compared with 440 g kg^{-1} for soya bean meal and 180-500 g kg^{-1} for meals of the other oilseeds (Anon 1957, 1974). Only a small amount of crude protein is present in the delinted cottonseed hulls (41 g kg^{-1}).

Table 1. 3 Chemical composition (g kg^{-1} DM) of oilseeds and their meals, and their energy values (MJ kg^{-1} DM) for ruminants (Sources: Van Soest *et al.* 1984; Coppock *et al.* 1987; Rust 1991)

Composition[a]	ME	NE*m*	NE*g*	NE*l*	Crude protein	NDF	ADF	Lignin	Ether extract	Ash
Cottonseed meal (solvent extracted)	13.0	7.9	5.2	7.7	456	260	190	60	141	70
Whole cottonseed (with lint)	16.0	9.9	6.9	9.3	239	390	290	160	208	48
Cottonseed hulls (with lint)	6.4	2.8	0.6	4.1	41	886	624	209	17	28
Soya bean meal (solvent extracted)	13.8	8.4	5.6	8.1	499	140	100	10	70	73
Soya bean seeds	15.1	9.3	6.4	8.8	428	-	100	-	58	55
Peanut meal (solvent extracted)	12.5	7.5	4.9	7.4	523	140	60	-	108	63
Peanut kernels (with skins and hulls)	15.9	-	-	-	236	-	-	-	211	32
Sunflower meal (solvent extracted)	6.3	4.0	0.4	4.0	259	400	330	120	351	63
Sunflower seeds	12.7	-	-	-	179	-	-	-	310	33

[a] ME, metabolisable energy; NE*m*, net energy for maintenance; NE*g*, net energy for growth; NE*l*, net energy for lactation; NDF, neutral detergent fibre; ADF, Acid detergent fibre.

The contents of the sulphur containing amino acids methionine and cysteine in CSM are comparable with those in soya bean meal, whereas the essential amino acids lysine, threonine, isoleucine and leucine are lower (Table 1. 4). The major amino acid deficiency in CSM is that of lysine. Fisher *et al.* (1971) studied conventional glanded and glandless CSM, and suggested that threonine, isoleucine and leucine became limiting after lysine and methionine. Chemical and heat treatment of feed protein can also affect the availability of amino acids, even though digestibility may not be impaired (Batterham 1992). According to Ashes *et al.* (1984) lysine, tyrosine and cystine are more sensitive to chemical and heat treatment than are other amino acids. Regular estimation of protein quality in heated oil meals is therefore warranted.

Cottonseed has a high fibre content owing to the thick seed coat. Cottonseed hulls contain neutral detergent fibre (NDF) of between 740 to 900 g kg^{-1} (Hsu *et al.* 1987; NRC 1989). In the regular, brown-coated varieties the hull fraction makes up between 35 to 40% of the seed by weight (Lawhon *et al.* 1977) depending on the

processing methods. Accordingly, the levels of NDF, acid detergent fibre (ADF) and lignin in CSM are high (Table 1. 3), being more than twice those in soya bean meal, particularly for lignin. However, variation occurs in seed-coat thickness, with light-colour-hulled strains containing less hull, and thus less fibre and lignin, compared with brown-hulled strains.

Table 1. 4 Essential amino acid composition (g kg^{-1} DM) of cottonseed meal and soya bean meal (Sources: Jones 1981; Knabe *et al.* 1989; Batterham *et al.* 1990)

	Cottonseed meal	Soya bean meal
Crude protein (N × 6.25)	360-480	425-428
Essential amino acid:		
Threonine	12-16	17-18
Valine	14-22	20-22
Cystine	8-14	8-15
Methionine	4-7	5-8
Isoleucine	11-16	19-20
leucine	19-29	30-34
Tyrosine	9-14	12-12
Phenylalanine	18-27	21-22
Histidine	11-17	12-14
Lysine	14-21	23-28

The oil (ether extract) content of CSM is between 5.0 to 140 g kg^{-1}, depending on the processing methods used (Jones 1981; Coppock *et al.* 1987) or approximately 210 g kg^{-1} in whole cottonseed (Lawhon *et al.* 1977; Coppock *et al.* 1987). When comparing processing methods, prepress solvent extracted meals contain low concentrations of oil, whereas the direct solvent extracted meals are higher in residual lipids. The lipid content of CSM is a major factor influencing its energy value.

1. 4. 2 Secondary Plant Compounds in Cottonseed Meal

Secondary plant compounds have been so named because they were originally thought not to be involved in the biochemical processes which form the basis of growth and reproduction in plants. Subsequently, it has been found that secondary compounds are produced by some plants as defence mechanisms against insect and fungal attack, and in others as a mechanism for restricting grazing by animals, thus ensuring survival of the plant (Barry and Manley 1987; Fitt *et al.* 1992).

Many secondary plant compounds have been recognised as being anti-nutritional or toxic factors for animals, particularly for monogastric animals. They can be either toxic to animals (e.g. gossypol, cyanide, nitrate and fluoroacetate), cause sub-clinical losses in productivity (e.g. some mycotoxins and high concentrations of condensed

tannins) or reduce mineral availability (e.g. phytic acid; Huisman 1989). However, some of them can either enhance nutritional value (e.g. low concentrations of condensed tannins in ruminant feeds; Barry 1989) or improve plant persistency (e.g. peramine, gossypol, tannin; Fitt *et al.* 1992).

The major secondary plant compounds in cottonseed are gossypol, tannins, cyclopropenoid fatty acids and phytic acid (Berardi and Goldblatt 1980; Frank 1987; Terrill *et al.* 1992a). The presence of gossypol and tannins is part of the host plant resistance mechanism in cotton for defence against attack by insects and pathogenic micro-organisms. Fitt *et al.* (1992) found that major variation in the diversity and concentration of terpenoids together with gossypol and tannins are toxic to *Heliothis* larvae.

The chemistry of and analytical methods for the secondary compound(s) in cottonseed, factors affecting their concentration in cottonseed and by-products, and the effect of the secondary compound(s) upon the nutritional value of cottonseed and by-products for animals will be reviewed in the following sections.

1.5 ANTINUTRITIONAL FACTORS IN THE COTTONSEED MEAL

Many feedstuffs contain substances which can produce different deleterious effects in the animal (Liener 1980, 1989; Marquardt 1989; Pusztai 1989). When these factors cause negative effects on growth, feed conversion efficiency and/or health, they are referred to as 'antinutritional factors' (ANFs). ANFs can be defined as "substances generated in natural feedstuffs by the normal metabolism of the species from which the material originated and which by different mechanisms exert effects contrary to optimum nutrition" (Gontzea and Sutzesu 1968). In this definition, however, fibre may also be classified as an ANF in monogastric animals. Therefore, one restriction is that ANFs have no feeding value (Huisman *et al.* 1990).

ANFs can be classified in various ways. In the following scheme, they are classified by their effects on nutritive value and/or the biological response in the animal (Chubb 1982; Huisman 1989): i) factors which have a depressive effect on protein digestion and on the utilisation of protein (trypsin and chymotrypsin inhibitors, lectins, saponins, polyphenolic compounds); ii) factors which have a negative effect on digestion of carbohydrates (amylase inhibitors, polyphenolic compounds, flatulence factors); iii) factors which have a negative effect on the utilisation of minerals (glucosinolates, oxalic acid, phytic acid, gossypol); iv) factors which inactivate vitamins or cause an increase in vitamin requirement (anti-vitamins).

The major naturally occurring anti-nutritional factors (ANFs) in CSM are gossypol (Ikurior and Fetuga 1984; Frank 1987), tannins (Ikurior and Fetuga 1984; Balogun *et al.* 1990; Terrill *et al.* 1992) and other potential anti-nutrients or toxic factors, such as cyclopropenoid fatty acids (CPFAs), phytic acid, phenolic acids, raffinose, aflatoxin and allergens (Anon 1973, Dabrowski and Sosulski 1984, Marsh *et al.* 1969).

1. 5. 1 Gossypol

The polyphenolic gossypol pigments (Boatner 1948; Altschul *et al.* 1958; Markman and Rzhekhin 1965; Bell and Stipanovic 1977; Frank 1987) are indigenous in the genus *Gossypium* and in certain other members of the order Malvales. In the cotton plant, they are contained almost exclusively within discrete bodies commonly called pigment glands, which are found in the leaves, stems, roots, and seed of cotton plants.

Gossypol is not uniformly dispersed throughout the seed, but is deposited in scattered structures, which can be seen as black specks in the stems, leaves and green bolls of the plant, and in the seed. Glands in the seed are ovoid structures containing 350-500 g/kg gossypol. They constitute about 24-48 g/kg of the weight of dehulled cottonseed kernels, and are 0.025-0.178 mm in diameter. The principal quality deficiencies of those cottonseed products are due to their contents of gossypol pigments (Berardi and Goldblatt 1980).

1. 5. 1. 1 Chemical Structure

Boatner (1948) reported the presence of at least 15 gossypol pigments or derivatives in extracts of cottonseed or cottonseed oil and meals, but only about eight have been isolated in more or less purified form and characterised. They include gossypol (yellow), diaminogossypol (yellow), 6-methoxygossypol (yellow), 6,6'-dimethoxygossypol (yellow), gossypurpurin (purple), gossyfulvin (orange), gossycaerulin (blue), and gossyverdurin (green).

Gossypol occurs in greater amounts in raw cottonseed than in cottonseed that has been subjected to moist heat treatment (cooked) during processing, whereas more gossypurpurin and gossyfulvin occur in the cooked seed. Gossycaerulin occurs almost exclusively in cooked cottonseed. Gossypol is converted to gossypurpurin during maturation and prolonged storage of the seed.

The predominant naturally occurring gossypol pigment, and the one that has been investigated far more thoroughly than any of the others, is the yellow pigment gossypol, $C_{30}H_{30}O_8$. Named by Marchlewski in 1899, gossypol is 1,1',6,6',7,7'-hexahydroxy-5,5'-

diisopropyl-3,3'-dimethyl[2,2'-binaphthalene]-8,8'-dicarboxaldehyde (Adams *et al.* 1960). The structure of gossypol, derived by Ory *et al.* (1983), is shown in Fig. 1. 3. The postulation of three tautomeric structures was necessary to explain many of the reactions of gossypol. Of the three tautomeric modifications of gossypol shown in Fig. 1. 4, structure (1a) represents the hydroxyaldehyde tautomer, (1b) the lactol tautomer, and (1c) the cyclic carbonyl tautomeric form.

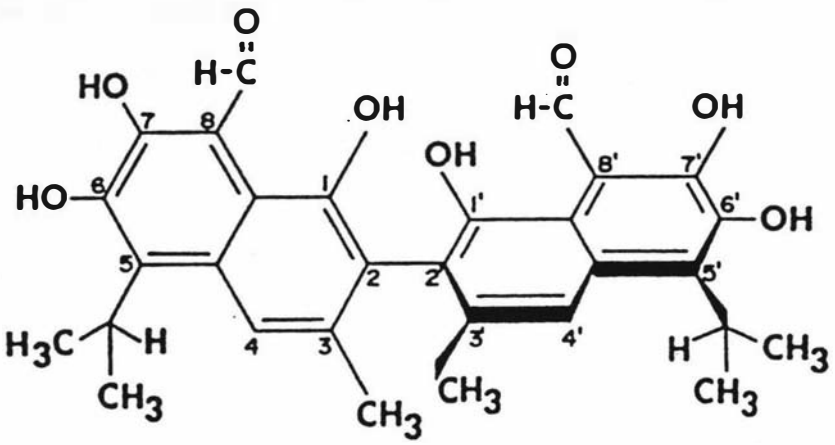
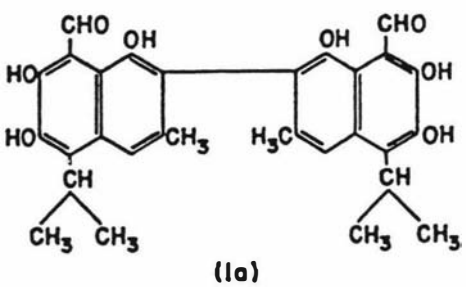
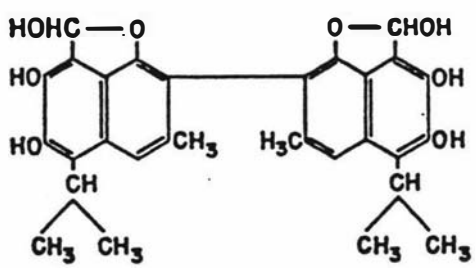


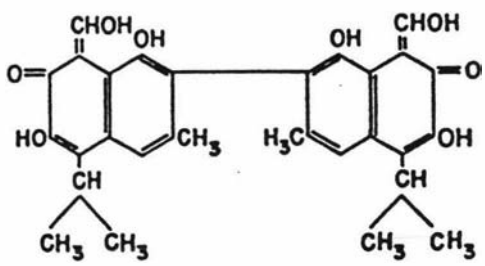
Fig. 1. 3 Chemical structure of gossypol (Source: Ory *et al.* 1983)



(1a)



(1b)



(1c)

Fig. 1. 4 Structures of the various tautomeric forms of gossypol (Source: Adams *et al.* 1938)

Gossypol (molecular weight 518.5 Da) is soluble in many organic solvents, and it is insoluble in low-boiling petroleum ether (bp 30-60°C) and in water. Crystalline gossypol and most of its solutions in organic solvents are photosensitive (Boatner 1948). Gossypol of mp 184°C is obtained upon crystallisation from ether, of mp 199°C from chloroform, of mp 214°C from ligroin (Campbell *et al.* 1937), and of mp 195°C from benzene (Shirley 1966).

Gossypol as it occurs in the cottonseed kernel is readily extracted by 70% aqueous acetone. When cottonseed is processed, the amount of gossypol that is extracted by this solvent decreases, and, in general, the more severe the processing conditions the less gossypol is extracted. However, all, or most, of the residual gossypol is rendered soluble if the sample is treated (hydrolysed) with oxalic acid. The gossypol extractable from the meal with 70% aqueous acetone (without prior hydrolysis) is defined as free gossypol, whereas that extractable after hydrolysis with oxalic acid is defined as total gossypol. The difference between the two values represents *bound* gossypol (Berardi and Goldblatt 1980; Frank 1987). In general, the amount of bound gossypol increases with severity of the processing conditions.

1. 5. 1. 2 Detoxification During Cottonseed Meal Manufacturing

Postulation of the three tautomeric forms of gossypol shown in Fig. 1. 4 was necessary to account for the numerous reaction products encountered in the establishment of the structure of gossypol and their characteristics. Thus, the hydroxyaldehyde tautomer (Fig. 1. 4a) is responsible for most of the normal aldehyde reactions of gossypol (O'Connor *et al.* 1954; Shirley 1966). The hexamethyl ether was formulated from the tautomer (Fig. 1. 4b) to account for the unusual stability to alkali, as contrasted to the ease of hydrolysis under acidic conditions with the loss of two methoxyl groups. The cyclic carbonyl tautomer (Fig. 1. 4c) accounts for the ready formation of anhydrogossypol and of identical Diels-Alder-type adducts of both gossypol and anhydrogossypol with dienes such as butadiene (Berardi and Goldblatt 1980). Comprehensive reviews on chemical reactions of gossypol were published by Adams *et al.* (1960), and Berardi and Goldblatt (1980).

As is well known, heating of oilseed meals during commercial processing, beyond inactivation of enzyme inhibitors and other toxicants, is detrimental to protein utilisation, particularly for nonruminant animals. The heating causes the formation of new-enzyme-resistant linkages within the protein molecule, so reducing its digestibility and the biological availability of some of the constituent amino acids (Ford 1973; Hurrell *et al.*, 1976). There are many possible cross-linking reactions, but one of the most likely seems to be the formation of new isopeptide bonds by reaction of the ε-amino group of

lysine with either the carboxyl group of aspartic or glutamic acids, or more probably with the amide groups of glutamine and asparagine. CSM is a major livestock protein supplement and, without heat processing, is a source of good quality protein (Martinez and Hopkins 1975). However, CSM is first limiting in lysine, and the reduced availability of lysine that occurs in normal commercial processing addition impairs the utilisation of CSM protein (Baliga and Lyman 1957; Batterham *et al.* 1990). The loss of available lysine in CSM is also due partly to its reaction with the pigment gossypol present in glanded cottonseed (Lyman *et al.* 1959). Gossypol in whole cottonseed is in the free form (Pons *et al.* 1953), but as a result of processing, both free and bound gossypol will be present in meal (Jones 1985). The binding of free gossypol is important because the free form can be toxic to animals if fed in high enough amounts.

1. 5. 1. 3 Effect of Gossypol on Animal Nutrition

The presence of gossypol in CSM presents two problems that are not easily resolved. The presence of high levels of free gossypol causes unfavourable physiological effects in monogastric animals, and the reaction between gossypol and protein during processing reduces protein quality, especially by a reduction in the biological availability of lysine for monogastric animals (Batterham *et al.* 1984, 1990b; Beech *et al.* 1991). The widely different processing methods used result in quite different protein qualities. Although many studies have been published concerning the role of gossypol as it affects the nutritive value of CSM, most are studies of gossypol plus gossypol-like pigments, and it is frequently difficult to relate the results of such studies to those conducted with purified gossypol. Additionally, the residual oil content of CSM, frequently ignored in the studies, may have an important effect.

The first published report of injury to animals from ingestion of CSM was that of Voelcker in 1858 (Berardi and Goldblatt 1980). Subsequently, cottonseed poisoning was variously attributed by early workers to the presence of choline, betaine, or nitrogenous bases of the ptomain type and to pyrophosphates (Withers and Carruth 1915). With the establishment by Withers and Carruth (1915) and others that purified gossypol had an effect similar to that of cottonseed when ingested by rabbits, rats, and pigs, despite much disagreement, they attributed all of the toxicity of cottonseed to gossypol.

General symptoms of gossypol toxicity are depressed feed intake and loss of body weight. Pathological symptoms are many and varied depending on animal species. In non-ruminants, death from gossypol effects is attributed to reduced oxygen-carrying capacity of the blood and hemolytic effects on erythrocytes (Danke and Tillman 1965). Gossypol can reduce succinic dehydrogenase and cytochrome oxidase activity in chick liver (Ferguson *et al.* 1959). Abou-Donia and Dieckert (1974) considered gossypol to

exert its toxic effects in animals and poultry by uncoupling respiratory-linked phosphorylation. Pigs and rabbits are more sensitive than are rats, poultry and dogs (Abou-Donia 1976). The pathological symptoms in some nonruminants attributed to toxicity of gossypol in cottonseed meal are given in Table 1. 5. Gossypol in CSM can also depress reproduction of monogastric animals. Free gossypol can cause decreased sperm motility and lower sperm concentration (Shandilya *et al.* 1982). Decreased sperm production has been attributed to damaged germ cells contained within the germinal epithelium. Free gossypol interferes with the expression of normal estrous cycles. Inhibition of pregnancy and embryo survival has been also reported (Eisele 1986). Possible mechanisms responsible for these effects of gossypol include endocrine imbalance. Data largely support an effect of gossypol on ovarian hormone secretion (Randel *et al.* 1992).

Table 1. 5 Antemortem and postmortem findings in some nonruminants attributed to chronic toxicity of gossypol in cottonseed meals (Source: Berardi and Goldblatt 1980)

Animal species	Antemortem symptoms	Postmortem symptoms
Rat	Loss of appetite; growth rate depression; diarrhea; anorexia; hair loss; anemia; reduced erythrocytes, hemoglobin, and packed cell volume; prevention of sperm mobility and production; reduced coital behaviour	Intestinal dilation & impaction; hemorrhagic congestion of stomach and intestines; congestion in lungs and kidneys; duodenal enteritis; degeneration of seminiferous tubules; dilated mitochondria in middle of spermatids
Rabbit	Stupor; lethargy; loss of appetite; diarrhea; hypoprothrombinemia; spastic paralysis; decrease in litter weights	Hemorrhages in small intestine, lungs brain, and leg bones; enlarged gallbladder; edema and impaction of the large intestine
Poultry	Loss of weight; decreased appetite; leg weak; lower hemoglobin and red blood cell count; lower protein and albumin/globulin ratio of serum; decreased egg size, egg yolk discoloration; decreased egg hatchability	Fluid in body cavities; enlargement of gallbladder and pancreas; liver discoloration; many vacuoles and foamy spaces in liver; ceroidlike pigment deposition in liver, spleen, and intestinal mucosa
Swine	Labored breathing; dyspnea; weak; emaciation; increase in glutamic oxaloacetic transaminase; weight loss; hair discoloration; altered electrocardiogram; diarrhea; reduced hemoglobin and hematocrit; deficiency of lymphatic cells	Widespread congestion and edema of many organs; fluid in body cavities; edematous bladder and thyroid gland; flabby, dilated heart with microscopic lesions; renal lipidosis; atrophied spleens; myocardial injury

During the early 1900s, ruminants were also considered to be susceptible to the toxic effects of gossypol (e.g., 'cottonseed meal poisoning'). It has, however, now been established that these symptoms were due to vitamin A deficiency in the diets rather than due to gossypol (Hale and Lyman 1948). Reiser and Fu (1962) indicated that ruminants avoid gossypol toxicity by detoxification as a result of free gossypol becoming bound to soluble proteins in the rumen. The gossypol most likely binds to the epsilon-amino

group of the amino acid lysine. This bond is not broken by the proteolytic enzymes secreted in the lower gut. The protein-bound gossypol complex was found to be physiologically inactive. It is known, however, that ruminants are susceptible to gossypol toxicity if the rumen detoxification process is somehow bypassed. Danke *et al.* (1965) and Morgan *et al.* (1988) have reported that studies conducted with sheep indicated that gossypol toxicosis could be induced by injecting or giving a bolus of large amounts of gossypol acetic acid; however, toxic symptoms were not caused by feeding cottonseed products to lambs.

Smalley and Bicknell (1982) reported case studies of gossypol toxicoses in mature dairy cattle fed relatively high amounts of ammoniated whole cottonseed (2.7 to 4.5 kg day^{-1}). Postmortem examination revealed oedema of the lungs and abomasum, presence of straw-coloured fluid in the thoracic and peritoneal cavities and hypertrophic muscle fibres in the heart. The postmortem findings are very similar to those found in nonruminant species. In dairy cattle fed excessive amounts of CSM (6.6 and 42.7 mg free gossypol kg^{-1} body weight day^{-1}), plasma concentrations of gossypol were elevated (1.7 and 1.8 vs 0 mg ml^{-1}) compared with control cows fed soya bean meal (Lindsey *et al.* 1980). Concentrations of free gossypol in livers were also higher in cows fed CSM (59 and 94 vs 0 mg g^{-1} of dry tissue). Other aberrations included lower haemoglobin, higher total plasma protein, greater erythrocyte fragility, and faster respiration rates in cows fed CSM diet than in control cows. This was further supported by findings of erythrocyte fragility in feeder lambs (Calhoun *et al.* 1990) and heifers (Gray *et al.* 1990) fed moderate amounts of cottonseed products containing free gossypol. Randel *et al.* (1992) concluded that ingestion of free gossypol at high levels can overwhelm ruminal detoxification and hence result in the absorption of quantities of free gossypol that may be potentially toxic.

Ruminant females are relatively insensitive to dietary gossypol from a reproduction point of view, but males show testicular damage. Young weaned bulls were fed whole cottonseed, which delivered 27 g day^{-1} of free gossypol or CSM which delivered 1.9 g day^{-1} free gossypol for more than 400 days. The bulls consuming whole seed were later in reaching puberty and bulls on both treatments had lower quality semen than bulls on a gossypol-free diet, and both sets of bulls recovered when gossypol was removed from the diet (Chase *et al.* 1989). As same as in the nonruminants, gossypol extensively damages germ cells contained with the germinal epithelium of the seminiferous tubules in the ruminant animals. (Chase *et al.* 1989). In a review by Nomeir and Abou-Donia (1985), nine studies were cited where gossypol did not cause females of several species to become infertile. There is limited data on the effect of gossypol on embryos. Zirkle *et al.* (1988) reported the result of the *in vitro* culturing of bovine embryos in fluid containing from 1-3 mg of free gossypol. This appeared to be a

physiological effect upon the embryo itself and not a mutational effect. Feeding high levels of free gossypol was also shown to delay the completion of pubertal processes (Chase *et al.* 1989; Jimenez *et al.* 1989).

1. 5. 2 Tannins

The term 'tannin' covers naturally-occurring plant polyphenols which combine with proteins and other polymers such as cellulose, hemicellulose and pectin, to form stable complexes (Mangan 1988). Chemical degradation studies have shown the plant tannins to be complex phenolic polymers containing aliphatic and phenolic hydroxyl groups and in some cases carboxyl groups (Haslam 1966). Tannins comprise a relatively small part of a large and diverse group of plant phenolics which range from simple C_7 - C_9 phenolic acids such as gallic and coumaric acids, through the C_{15} flavanoids to the highly-polymerised inert lignins. The chemistry of the tannins has been extensively reviewed (Swain 1979; Haslam 1981).

1. 5. 2. 1 Chemical Structure

The tannins have been classified into two groups based on structural types (Freudenbery 1920) and reactivity toward hydrolylic agents (Haslam 1966). They are the hydrolysable tannins (HT) and the condensed tannins (CT).

HT have a central carbohydrate core which serves as a polyalcohol to which a number of phenolic carboxylic acids are bound by ester linkages. Because of the polyester type of structure the molecule may be hydrolysed into simpler fragments. The HT can be further subdivided into gallic acid or *m*-digallic acid (gallotannins) or hexahydroxydiphenic acid (ellagitannins; Fig. 1. 5). The gallotannins are not widespread, but tannic acid is a well-known example (Haslam 1979).

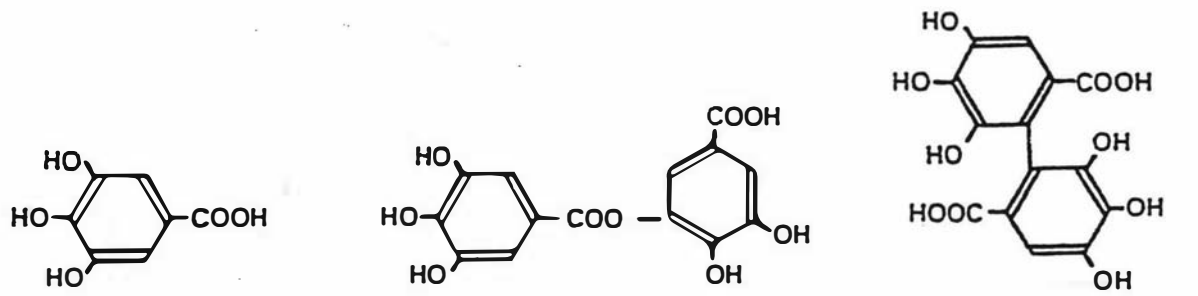


Fig. 1. 5 Structure of Gallic acid, *m*-Digallic acid and Hexahydr-oxydiphenic acid

CT are the most widespread and typical of the plant tannins and have no carbohydrate core, but occur as a range of polymers (procyanidins), and are polymers of flavan-3-ols (catechins) and flavan-3,4-diols (Fig. 1. 6; Swain 1965). Condensed tannins are not readily degraded by acid treatment but polymerise to form amorphous phlobaphenes or 'tannin-reds' (Quesnel 1968; Roux 1970). Similarly heating in acid solution converts CT to the corresponding anthocyanidins and brown 'phlobaphene-like' polymers (Swain and Hillis 1959). A typical structure of a CT isolated from grape, cranberry and sorghum grain is shown in Figure 1. 6 (Haslam 1977).

The molecular weight of the CT is usually in the range 500-3,000 Da (Foo *et al.* 1982). Bate-Smith (1973) found that CT have maximum activity when their molecular masses are at least 1134 Da, while Kumar and Singh (1984) demonstrated a decrease in solubility of CT when their molecular weight is over 5,000 Da.

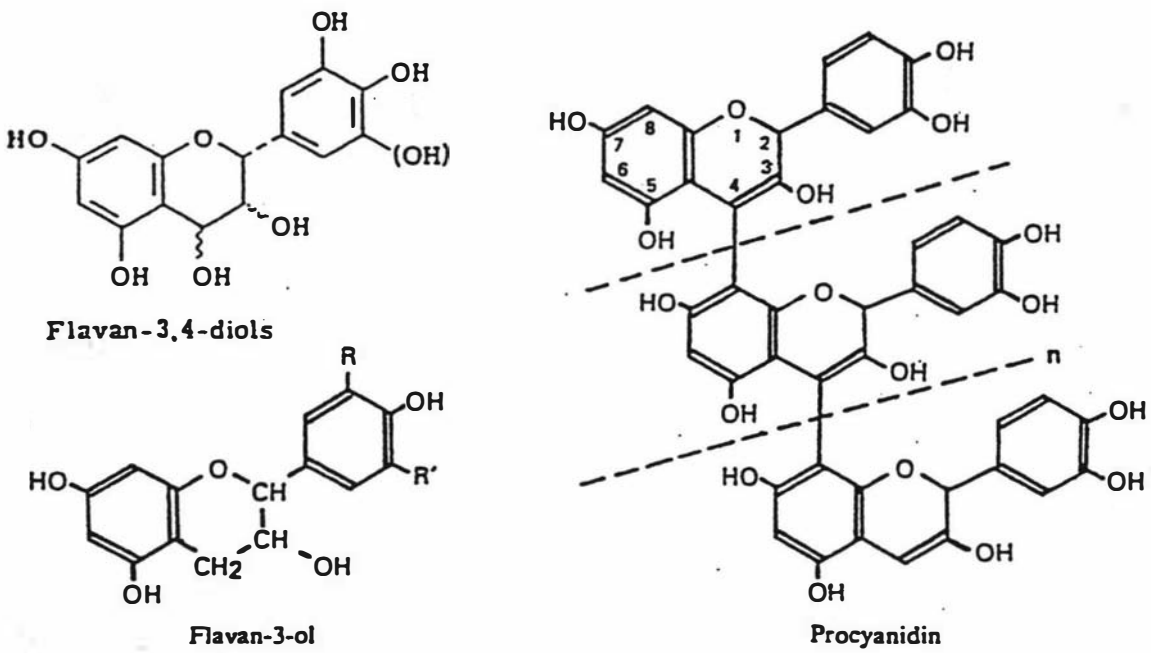


Fig. 1. 6 Chemical structure of Flavan-3,4-diols, Flavan-3-ol and Procyanidin (condensed tannin)

1. 5. 2. 2 Reactions with Protein and Carbohydrate

CT can form weak reversible bonds with a range of compounds, including cellulose and protein, the strength of which is a function of the molecular weight (Roux 1955, 1958)

and the molecular configuration of the phenol (Roux and Evelyn 1958; Roux *et al.* 1961; Roux 1963).

Russell *et al.* (1968) and Haslam (1974) found that tannins have a greater affinity for protein than for cellulose, which has been attributed to the strong hydrogen bond affinity of the carbonyl oxygen of the peptide group. Three other types of bonds have been suggested to participate in the formation of CT-protein complexes: i) covalent links formed by oxidation of polyphenols to quinones followed by condensation with a nucleophilic group (-NH_2, -SH, -OH) in the protein (Loomis 1974; Haslam 1979); ii) ionic bonds between the cationic site of the protein molecule and the phenolate anion (Loomis 1974), and iii) hydrophenolic interaction between the aromatic ring structure of phenolic compounds and hydrophobic regions of protein (Loomis 1974; Hagerman and Butler 1980).

Several factors can influence CT-protein interactions. The CT-protein interactions are largely dependent on pH. The hydrogen bond of CT-protein complexes from sainfoin are stable at pH 3.5-7.0, but are unstable at pH<3.0 and pH>8.0 (Jones and Mangan 1977). Hagerman and Butler (1978) reported that precipitation was greatest at a pH within ±1 unit of the isoelectric points of protein. The CT composition and their molecular size or weight also influences the protein precipitating properties of CT. Bate-Smith (1973) reported that the minimum molecular weight for effective protein precipitation is about 350 Da. Horigome *et al.* (1988) showed that for a given CT, the protein-precipitating capacity increased with the increasing degree of polymerisation. Proteins having open, loose conformation, high molecular weight and high contents of proline and other hydrophobic amino acids have a high affinity for CT (Asquith and Butler 1986).

CT can also react with carbohydrates (or polysaccharides), however, the reactions are less well understood than with protein. McLeod (1974) showed that the precipitating capacity of CT with protein was stronger than with carbohydrates. Observation showed a similar pattern of affinity to those noted for proteins, in particular the affinity of polyphenols for sites and environments which permit the hydrophobic interactions to develop (Haslam 1989).

Polyethylene glycol (PEG) can specifically combine with CT to form CT-PEG complexes. The bonds in these complexes are stronger than the bonds in CT-protein complexes. Thus PEG can be used to displace protein from the CT-protein complexes (Jones and Mangan 1977; Barry and Manley 1986). Therefore, effects of CT can be quantified by comparing controls (CT acting) with PEG treatments (CT inactivated). This reaction provides a unique way to study the effect of CT without affecting other

nutrients in the diet (Oh *et al.* 1980; Waghorn *et al.* 1987a; Barry 1989; McNabb *et al.* 1993; Wang 1995).

1. 5. 2. 3 Analytical Methods for Tannin Detection

A major problem in studying tannins is the tendency of phenols to oxidise during sample preparation and extraction (Gundagni *et al.* 1949; Craft 1961). Chopping or macerating the fresh plant material or wilting in the field was found to reduce the extractable CT content in forage (Lyford *et al.* 1967). Drying forage samples in a forced draught oven can also cause oxidative changes to tannins (Goldstein and Swain 1963). Suitable reducing agents have been shown to increase yields of tannins when added to the extraction solvents (Khanna *et al.* 1968). In some species of legumes such as sainfoin the level of tannin extraction can be improved by fine milling (100 mesh) the leaves (Bate-Smith 1973b).

Various methods are available for quantification of polyphenolic compounds. The methods are based on their chemical properties, for example, Folin-Ciocalteu, Folin-Denis, Prussian Blue, ferric ammonium citrate, and formaldehyde-HCl methods are used to determine total phenols. The vanillin-HCl and butanol-HCl methods are specific to CT and were developed to measure extractable CT. Colorimetric methods have been discussed at length by Deshpande *et al.* (1986). Lately, attention has been focused on quantification of tannins based on their operational property viz. binding/precipitation of proteins because the nutritional, physiological and ecological roles of tannins are attributed to the complexing of tannins with proteins (Deshpande *et al.* 1986; Mole and Waterman 1987a). Other methods employing nuclear magnetic resonance (NMR), near infra-red (NIR) and HPLC (Makkar 1989; Haro *et al.* 1989; Okuda *et al.* 1989) are less frequently used because of high cost and the complexities involved.

Different chemical methods measure different classes of phenolic compounds. Therefore, these methods besides providing information on levels of tannins in a sample also help in elucidating their structure. Different methods require different tannins as standards and the values obtained for the sample are expressed relative to the standard. Two types of standards are used: absolute standard, obtained by purifying the standard from the plant of interest; and relative standard, commercially available tannins. The use of absolute standard, although desirable, is not always practicable, due to laborious methods of obtaining well characterised tannins (Hagerman and Butler 1989). The relative standard, such as tannic acid for methods based on the oxidation-reduction principle and protein precipitation, catechin for vanillin-HCl assay and quebracho tannin for butanol-HCl method, has been widely used (Makkar 1993). However, various impurities are present in the sample of tannic acid and quebracho tannins (Hagerman and

Butler 1989). The commercially available purified and well characterised CT standards are very important in polyphenolic research. Use of such standards ensures reproducibility within and between laboratories and allows meaningful comparison of values obtained by different laboratories.

Methods generally used by our laboratory are the vanillin-HCl assay and a modification of the butanol-HCl colorimetric procedure (Porter *et al.* 1986), as described by Terrill *et al.* (1992a). In the latter method, a technique for separation of total CT into extractable, protein-bound and fibre-bound fractions was developed, and the butanol-HCl procedure was also modified to use aqueous extracts of these three fractions. Developments or modifications include a more rapid procedure for obtaining extractable CT, the use of different standard curves for extractable and bound CT, the use of aqueous plant extracts and an evaluation of the effects of boiling time. The fractionation of CT into extractable, protein- and fibre-bound CT is important for some kinds of studies, particularly for studies involving protein concentrate meals, since the CT in protein concentrate meals, unlike CT in forage, is mainly in the protein- and fibre-bound forms (see Table 1. 6). This procedure has been used to measure contents of extractable and bound CT in many samples of different feedstuffs (Terrill *et al.* 1992a; Wang *et al.* 1994), with the standard being CT extracted from *Lotus pedunculatus*.

Table 1. 6 Extractable and bound condensed tannin contents (g kg^{-1} DM) of a range of forages and protein concentrate meals, determined by the butanol-HCl procedure.

	Condensed Tannins				
	Extract-able	Protein bound	Fibre bound	Total	Reference
Legumes					
Canary clover	83.0	54.0	6.0	143.0	Terrill *et al.* (1992a)
Big trefoil	61.0	14.0	1.0	76.0	" "
Sulla	33.0	9.0	3.0	45.0	" "
Birdsfoot trefoil	27.1	6.1	1.8	35.0	Wang et al. (1994)
Grasses					
Perennial ryegrass	1.1	0	0	1.1	Terrill *et al.* (1992a)
Yorkshire fog	1.1	0.3	0.4	1.8	" "
Protein concentrate meals					
Cottonseed	2.1	10.0	3.9	16.0	" "
Rapeseed	0.7	3.7	1.5	5.9	" "
Soya bean	1.0	0	0	1.0	" "

1. 5. 2. 4 Effects of Tannins on Animal Nutrition

Numerous studies have been conducted on the effect of tannins in feedstuffs on animal performance. Some of these have been carried out with isolated tannins from feedstuffs or with "standards" of commercial tannins, such as tannic acids, which were thought to

be representative of tannins in a number of feedstuffs. Most studies, however, were carried out with raw or fractionated feedstuffs (e.g. hulls of legume seeds) of the same plant species and forages containing different levels of tannins as analysed by one of the available analytical methods. In these studies the effects or differences found were fully or partly related to the differences in tannin level in the experimental diets.

1. 5. 2. 4. 1 Monogastric Animals

Ikrior and Fetuga (1984), Balogun *et al·* (1990) and Terrill *et al.* (1992a) have detected CT, as a further group of secondary compounds present in commercially produced CSM. CT are likely to further reduce protein nutritional value in diets for monogastric animals (Huisman *et al.* 1990; Longstaff and McNab 1991; Jansman 1993).

Table 1. 7 summarises the nutritional effects of tannins in several feedstuffs on the performance of monogastric animals, and upon nitrogen, amino acid and energy digestibility in these species. Some general observations presented in Table 1. 7 are: i) it has not been conclusively demonstrated that tannins will reduce feed intake in monogastric animal species; ii) tannins in diets generally will reduce weight gain and impair feed conversion efficiency in growing animals; iii) tannins reduce the apparent digestibility of protein, amino acids and, to a lesser extent, energy. The large number of variables that tend to modify the harmful effects of tannins limits the usefulness of direct comparison among the different studies.

Tannins are known to have a bitter or astringent taste and may reduce palatability and hence feed intake. In contrast, it has been suggested that a slightly astringent taste increasesthe palatability of feed and stimulates feed intake (Gupta and Haslam 1980). The physical basis for astringency may be that tannins bind and perhaps precipitate salivary proteins and inactivate enzymes (Harborne 1976). This would reduce the lubricating property of saliva, give the mouth a feeling of dryness and affect the animals' ability to swallow the food (Mole 1989). A second, more direct way by which tannins affect feed palatability may be that tannins directly bind to taste receptors (Mole 1989).

Glick and Joslyn (1970) and Vohra *et al.* (1966) showed a reduction in feed intake in rats and chickens due to the supplementation of tannic acid. In contrast, an increased feed intake was found in chicks fed sal seed, a seed that contains high levels of HT (Zambade *et al.* 1979). The opposite results, however, were found by Ahmed *et al.* (1991). The level and type of tannins as well as differences among animal species may explain the contrasting results with respect to the effect of tannins on feed intake. In natural ecosystems there is clear evidence that different animal species select feeds of vegetable origin on the basis of their tannin contents and that the normal or accepted

Table 1. 7 The examples of tannins and their nutritional effects in monogastric species

Source	Tannin level (g/kg)	Effect[1]	(% changes)	References
Poultry:				
Sorghum	0/19.2 D	ADG	-9.0	Dale *et al.* (1980)
		FC	+20	
Sorghum	0.8/19.1/28.3 D	DCn	-44.4	Mitaru *et al.* (1985)
Faba bean	0/0.6/1.7 S	FI	no effect	Jansman *et al.* (1993b)
		FC	no effect	
Faba bean	low(1)/high(1)	trDCn	-18 (Max)	Martin-Tanguy *et al.* (1977)
Pigs:				
Sorghum	low(2)/high(2) 1.0/1.0/36/38 S	ADG	-5.4	Myer & Gorbet (1985)
		FI	+5.9	
		FC	-14.6	
Sorghum	low(2)/high(2) 0.8/19.1/28.3	il.DCd	+0.5	Mitaru *et al.* (1984)
		il.DCn	-6.9	
		DCd	+7.4	
		DCn	-10.6	
Faba bean	low(2)/high(2) 10/15/17 S	ADG (23-60 kg)	-7.5	Grosjean & Castaing (1984)
		FC	+9.7	
		ADG (60-103 kg)	-6.4	
		FC	+6.7	
Faba bean	low(1)/high(3) 0.6/1.2/1.5 S	il.DCn	-13.2	Jansman *et al.* (1993c)
		il.DCaa	-10.7	
		DCn	-6.8	
Rats:				
Sorghum	5.0/7.0/13 S	FI	no effect	Muindi & Thomke (1981)
		DE	-4.0	
		DCn	-15.4	
		DCnfe	-3.0	
Carob tannins		ADG	-22.9	Tamir & Alumot (1969)
		FI	-4.7	
		FC	+23.3	
		DCn	-11.8	
Faba bean	0/14.1/19.9 D	FI	-46.3	Jansman *et al.* (1993a)
		DCaa	-33.3	
Faba bean	low(1)/high(1)	trDCn	-3.1	Ford & Hewitt (1979)
		NPU	-11.8	

1 Difference between the value for the high-tannin group(s) and the control or low-tannin group(s).

D or S : level in diet or source
ADG : average daily gain
FI : feed intake
FC : feed conversion efficiency
DE : digestible energy (units)
NPU : net protein utilisation
tr.DCn : true faecal N digestibility (units)
il.DCd : apparent ileal DM digestibility (units)
il.DCn : apparent ileal N digestibility (units)
DCd : apparent faecal DM digestibility (units)
DCnfe : apparent faecal N-free extract digestibility (units)
DCn : apparent faecal N digestibility (units)
DCaa : apparent faecal amino acid digestibility (units)

tannin level in the diets of animals in their natural environment differs among species (Mole 1989).

Dietary tannins reduced apparent protein and amino acid digestibilities (Ford and Wewitt 1979, Jansman *et al.* 1993a, 1993c). Also a reduced energy digestion in pigs (Duee *et al.* 1979) and in poultry (Hersted 1979) has been observed. The dietary tannins also affect vitamin and mineral metabolism (Salunkhe *et al.* 1990). However, these effects seem to be less important than the effects on protein digestion. The reduced apparent protein digestibility of tannin-rich feeds may be explained either by a direct binding of tannins to dietary protein, by a reduced activity of protein-degrading enzymes (Longstaff and McNab 1991; Griffiths 1979, 1981), or by increased secretion of endogenous proteins (digestive enzymes, mucus or mucosal cells; Mangan 1988; Marquardt 1989).

Griffiths (1981) determined the activity of digestive enzymes in intestinal contents of rats fed diets containing hulls of high- and low-tannin varieties of faba bean. Activities of trypsin, chymotrypsin and α-amylase were reduced in animals fed the high-tannin diet. Tannin-containing extracts from rapeseed (Yaper and Clandinin 1972), green gram and ripe carobs (Tamir and Alumot 1969), chickpeas and pigeon peas (Singh 1984) have also been found to impair the *in vitro* activity of digestive enzymes. Griffiths and Moseley (1980) suggests that dietary tannins may also increase pancreatic secretion of digestive enzymes, and in some animal species tannins may stimulate pancreatic secretion in a manner analogous to that of protease inhibitors from legume seeds (Liener 1989). This could explain why dietary tannins in some cases increase activities of lipase in intestinal digesta.

1. 5. 2. 4. 2 Ruminant Animals

Tannins are present in many forages and fodder tree leaves, agricultural by-products and several agricultural wastes, where they often provide a natural protective system against attack by micro-organisms, insects or against being eaten by ruminants. High concentration of CT in plant material restricted the voluntary intake and reduced both body gain and wool growth rates of sheep (Pritchard *et al.* 1988, 1992). Barry (1985) found oral supplementation of PEG to lambs grazing *L. pedunculatus* (76-90 g extractable CT kg^{-1} DM) increased live weight gain and wool growth by 41-61 g d^{-1} and 23 mg $(100\ cm^2)^{-1}$, respectively, indicating that these levels of CT were limiting animal production.

Barry *et al.* (1986a) and Barry (1989) suggested that for the forage diets, levels of extractable CT in the range 20-40 g kg^{-1} DM may be beneficial in terms of dietary

protein utilisation. Studies (Ulyatt *et al.* 1977; John and Lancashire 1981) showed that animal performance was improved by feeding low CT-containing forages compared with other non CT-containing forages, such as red clover, lucerne and perennial ryegrass. However, these results cannot be considered as solely due to CT in the diets, since the forages differed in several other aspects. Terrill *et al.* (1992b) using oral PEG supplement to the grazing sheep showed that the action of CT in sulla (total CT 40-50 g kg^{-1} DM) increased live weight gain and wool growth. They, however, suggested that PEG supplement might not completely render all CT inert, and the animal response to the CT might not be fully expressed.

Barry and Manley (1984) established a significant linear relationship between dietary CT concentration and duodenal non-ammonia nitrogen (NAN) flow in sheep fed forage diets (Fig 1. 7). NAN flow out of the rumen per unit N eaten increased as forage extractable CT concentration increased, and it became unity at the value of 40 g CT kg^{-1} DM (i.e. undegraded dietary N+microbial N flowing at the duodenum equalled the N eaten). Waghorn *et al.* (1987a, 1994) showed that the abomasal amino acid flow was increased significantly by CT, particularly for essential amino acids, when sheep were fed *Lotus* species (Table 1. 8). McNabb *et al.* (1993) found that the CT in *L. pedunculatus* (extractable CT 55 g kg^{-1} DM) reduced the proteolysis of forage sulphur amino acids in the rumen.

Unlike the effect of CT upon rumen protein degradation, there was no clear relationship between the apparent post-ruminal digestibility of N and CT concentration. However, there do appear to be differences between concentrations of CT upon the apparent post-ruminal digestion of essential amino acids. The low concentration of CT in *L. corniculatus* (22 g extractable CT kg^{-1} DM) increased the apparent absorption (proportion of intake) of essential amino acids in the small intestine, whereas the high concentration of CT in *L. pedunculatus* (55 g extractable CT kg^{-1} DM) decreased the apparent digestibility of essential amino acids (excluding methionine and cysteine; Waghorn *et al.* 1987a, 1994; Table 1. 8) in the small intestine and resulted in no increase in net absorption. The effect of CT on post-ruminal N digestion may be a product of both concentration and sources of CT. The depression of apparent amino acid digestibility in the small intestine of sheep fed *L. pedunculatus* may be due to slow release of amino acids from the CT:protein complex, which will affect the true digestibility, and/or increase endogenous protein losses.

The mechanism of CT-carbohydrate formation is similar to that of CT-protein, but CT react more strongly with protein than with carbohydrate and compared with CT-protein, the CT-carbohydrate complex is less stable (McLeod 1974). In forage materials, usually only a small amount of CT binds with carbohydrate either before or after animal

chewing (Terrill *et al.* 1992a). Therefore it is reasonable that the interaction between CT and carbohydrate has a lower influence on the digestion of carbohydrate. However, this situation may not apply to some protein concentrate meals since the majority of CT in those meals is in bound forms, either with protein or with fibre (Table 1. 6). Barry and Manley (1984) found that the high CT concentration (106 g kg^{-1} DM) in *L. pedunculatus* reduced readily fermentable carbohydrate, structural carbohydrate and lignin digestibility in the rumen, but this was compensated for by increased digestibility in the intestine (Table 1. 9). In contrast, the low CT concentration (22 g kg^{-1} DM) in *L. corniculatus* had no effect on the digestion of both water soluble carbohydrate and structural carbohydrate (Ulyatt and Egan 1979; Waghorn *et al.* 1987b).

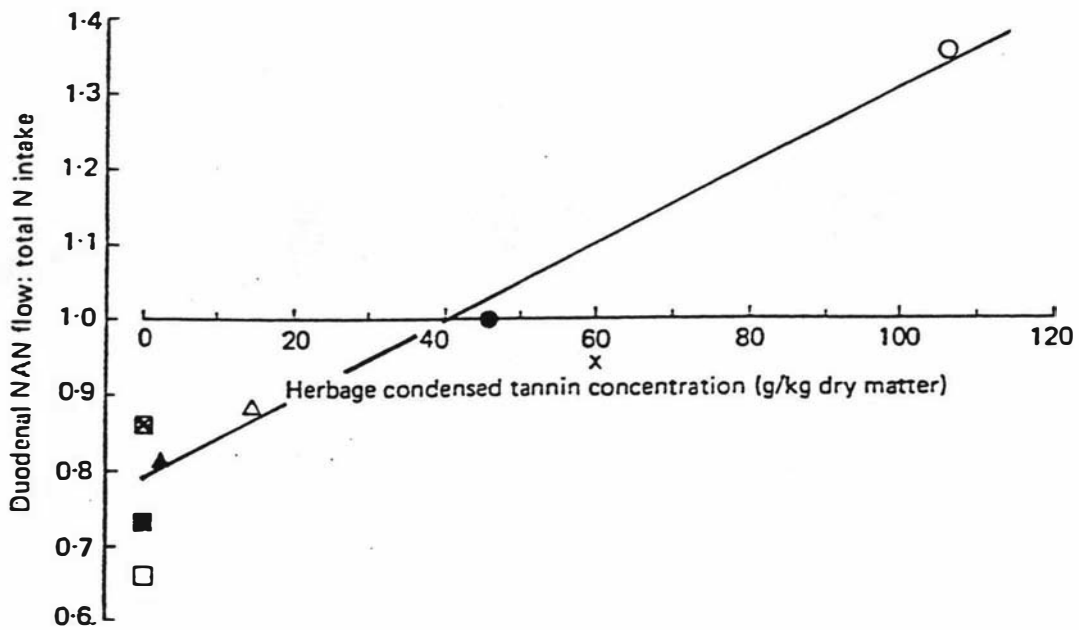


Fig. 1. 7 Duodenal non-ammonia nitrogen (NAN) flow per unit total N intake as a function of forage condensed tannin (CT) concentration in sheep fed on *Lotus* species. O, high extractable CT (106g kg^{-1} DM) *Lotus pedunculatus*; ●, low extractable CT (46g kg^{-1} DM) *Lotus pedunculatus*; Δ, high extractable CT (14.5 g kg^{-1} DM) *Lotus corniculatus*; ▲, low extractable CT (2.5 g kg^{-1} DM) *Lotus corniculatus* (John & Lancashire 1981); ⊠, short ratetion ryegrass; □, perennial ryegrass; ■, white clover (MacRae & Ulyatt 1974) and ×, sainfoin (Ulyatt & Egan 1979; Source: Barry and Manley 1984)

Table 1. 8 The effect of condensed tannins (CT) upon the digestion of essential amino acids in sheep fed fresh *Lotus corniculatus* (22 g extractable CT kg^{-1} DM) and *Lotus pedunculatus* (55 g extractable CT kg^{-1} DM; Sources: Waghorn *et al.* 1987a, 1994)

	L. corniculatus		*L. pedunculatus*	
	Control	PEG	Control	PEG
Amino acid intake (g d^{-1})	98.9	98.9	103.2	116.8
Abomasal flow (g d^{-1})	84.7	55.5	121.1	105.6
Proportion of intake	0.86	0.56	1.17	0.90
Apparent absorption from small intestine (g d^{-1})	58.8	36.2	81.4	83.5
Proportion of intake	0.59	0.37	0.79	0.72
Proportion of abomasal flow	0.67	0.67	0.67	0.79

Table 1. 9 Ruminal and post-ruminal digestion of readily fermentable carbohydrate (RFC) and structural carbohydrate in sheep fed *Lotus pedunculatus* differing in extractable condensed tannin (CT) content (Source: Barry and Manley 1984)

	Low-CT *Lotus* (46 g kg^{-1} DM)			High-CT *Lotus* (106 g kg^{-1} DM)		
	RFC	Hemi-cellulose	Cellulose	RFC	Hemi-cellulose	Cellulose
Apparent digestibility						
Proportion of intake	0.95	0.73	0.78	0.93	0.56	0.63
Ruminal digestion						
Proportion of intake	0.80(0.93)[1]	0.44(0.58)	0.69(0.69)	0.78(0.93)	0.21(0.42)	0.53(0.54)
Proportion of total digestion	0.84	0.61	0.89	0.83	0.38	0.85
Post-ruminal digestion						
Proportion of intake	0.15(0.06)	0.28(0.15)	0.09(0.09)	0.16(0.06)	0.35(0.14)	0.10(0.09)

1 Number in parentheses are predicted from the equation of Ulyatt & Egan (1979); derived with non-tannin-containing fresh forages.

Compared to the effect of CT in forages, the effect of CT in protein supplement meals on animal production is less understood. Lambs fed tannin-treated or untreated soya bean meal for a 16 day preliminary period gained 217 and 117 g d^{-1}, respectively (Driedger and Hatfield 1972). The N retention was 7.5 and 10.6 g d^{-1} for untreated and treated meal. However, the effect of the treatment on stability of the pellets may have been responsible for some of the beneficial effects of tannin. Balance studies with sheep fed skim milk powder treated with chestnut tannin showed a small decrease in digestible N (68.2 vs 72.0%), but a significant improvement in N retention (23.6 vs 16.1%; Delort-Laval *et al.* 1972). Zelter *et al.* (1970) reported that treatment of peanut, soya bean, linseed, rapeseed and sunflower seed meal, dried skim milk and casein with aqueous solutions of chestnut tannin prevented their degradation by rumen microorganisms.

Ehoche *et al.* (1983) compared cottonseed cake treated with *Bagaruwa* tannin at 0, 50 and 100 g kg^{-1}. They found that all levels of tannin treatment of cottonseed cake reduced ammonia accumulation in the rumen. Average daily gains were increased for the 50 g kg^{-1} level, but decreased for 100g kg^{-1} group. The treated cake reduced N digestibility by 3% units in the 100g kg^{-1} tannin group, and N retention was improved by 50g kg^{-1} but not 100g kg^{-1} level of tannin treatment.

1. 5. 2. 5 Animal Defensive Response towards Dietary Tannins

A number of herbivorous species consume tannin-rich feedstuffs as a part of their natural diet, without showing severe toxic or otherwise detrimental effects. The effect of tannins is minimised by physiological (Bernays *et al.* 1989) or behavioural (Roy and Bergeron 1990) adaptations that neutralise dietary tannin. The major physiological adaptation in mammals that has been identified so far is production of salivary tannin-binding proteins (Mehansho *et al.* 1987b; Austin *et al.* 1989; Robbins *et al.* 1991; Hagerman and Robbins 1993).

When rodents such as rats are fed tannin-rich diets, they show an initial loss of body weight, and then the animals start to gain weight again (Glick and Joslyn 1970b; Mehansho *et al.* 1983). The latter authors found that in the adapted animals the parotid glands had undergone dramatic hypertrophy, accompanied with an increase in production of a series of proline-rich proteins (PRPs). It was subsequently shown that these proteins have a very high binding affinity for tannins, being ten times higher than the affinity of BSA (Butler *et al.* 1982). It is assumed that the secreted PRPs in animals receiving a tannin-rich diet act as binding agents for tannins, thereby preventing other harmful and antinutritional effects (Butler *et al.* 1986). The PRP response due to dietary tannins was also found in mice (Mehansho *et al.* 1985), but not in the hamster (Mehansho *et al.* 1987a).

The mechanism of PRP induction by dietary tannins is most likely mediated via β-receptors, but the exact mechanism is unknown (Butler *et al.* 1986; Jansman 1993). Other functions of these proteins have been described (Bennick 1982) or suggested (Mole *et al.* 1990). Besides the adaptive mechanism of the parotid glands of rodents towards dietary tannins, no information is available on adaptive mechanisms in other simple-stomached species, including those important farm animals, such as pigs and poultry.

The high tannin diets of many browsing ruminants may be associated with active defences against plant tannins. Increased secretion of salivary proteins that bind and,

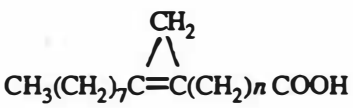
thereby, neutralise tannins may also be one defence used by browsing ruminants. Provenza and Malechek (1984) suggested that salivary or plant protein consumed by goats might bind with as much as 50% of the dietary tannins during ingestion. Robbins *et al.* (1987) conducted a study to measure binding capacities of saliva in ruminants with different feeding habits, and parotid salivary gland size has been measured in several ruminants (Hofmann 1973; Kay *et al.* 1980). Parotid salivary glands (g kg^{-1} body weight) are three times larger in browsing ruminants (i.e. goat and deer) than in domestic ruminants (i.e. sheep and cattle). Those authors suggested that size of different parotid salivary glands are best explained by the range in tannin intake and the requisite production of salivary proteins.

The production of glycoproteins for lubrication (Clifford 1986) and proline-rich salivary proteins to bind dietary tannins, the rapid disintegration of leaf cell walls and passage from the rumen (Spalinger *et al.* 1986), and the higher rumen fermentation rate (Hungate 1959; Prins and Geelen 1971) in browsing ruminants are essential for tree and shrub leaves to be useful food for animals.

1. 5. 3 Other Antinutritional Factors

1. 5. 3. 1 Cyclopropenoid Fatty Acids

The cyclopropenoid fatty acids (CPFA's) are acids structurally related to oleic acid that contain a cyclopropane ring at the site of the double bond. Two of these, malvalic acid and sterculic acid, occur naturally as esters in plants of the Malvales order, which includes all of the Gossypeae (Frank 1987).



n=6, malvalic acid

n=7, sterculic acid

The CPFA content varies significantly with cultivar and with location (Cherry *et al.* 1978), and is much higher in immature seed. The level of CPFA's is highest in *G. arboreum* species and lowest in *G. barbadense* species. Examination of individual seeds shows that 75% of the CPFA is located in the axial tissue, almost half of it in the radicle tip. The axis and radicle tip together account for only 5% of the weight of the kernel (Fisher and Cherry 1983). The ratio of malvalic to sterculic acid in cottonseed is usually about 2.50-2.75:1, and is independent of location in the seed. Malvalic acid is not as physiologically active as sterculic acid (Jones 1981). Cottonseed also contains dihydromalvalic and dihydrosterculic acids, the immediate precursors of malvalic and

sterculic acid in the biosynthetic pathway from oleic acid (Bianchini *et al.* 1981), but these are of no concern since the saturated acids are physiologically inactive whether as denaturation inhibitors or cocarcinogens (Jones 1981).

The level of the CPFA's ranges from 21 to 153 ppm (0.021-0.153 g kg^{-1}) in CSM and from 5 to 15 g kg^{-1} in crude cottonseed oil (Levi *et al.* 1967; Martinez *et al.* 1970). The more oil left in the meal, the higher the CPFA content. The CPFA content of CSM can be reduced to 5-10 ppm by successive extraction with solvent mixtures such as acetone-hexane-water. Hexane alone is less efficient (Reilich *et al.* 1968). Chemical inactivation with anhydrous sulphur dioxide reduces the CPFA content of CSM by over 90%; organic acid and sulfhydryl compounds are only partially effective and require the use of solvents (Reilich *et al.* 1969). In practice, the CPFA content is minimised by processing only mature seed and by extracting the oil as thoroughly as possible.

The CPFA's were of little concern other than for their ability to discolour egg whites when cottonseed oil was added to the diets of laying hens, but it was later discovered that both compounds are carcinogenic to rainbow trout, particularly, when combined with aflatoxin B_1 (Anon 1973). An excellent review of the biological effects of various cyclopropenoid compounds is available (Phelps *et al.* 1965), so only general comments in regard to the effects of these compounds on animal production will be made here. Although it is possible to demonstrate adverse effects of such compounds on the growth rate of broilers, it requires extremely high levels which would not normally be encountered from CSM *per se* (Waldroup 1981). However, the CPFA's may cause detrimental effects in laying hens, including disturbed lipid metabolism and release of iron from yolk, through their effects on membrane permeability. These acids are also inhibitors of desaturase enzymes, which if protected from rumen hydrogenation in lactating cows, increase the proportion of stearic acid at the expense of oleic acid (Cook *et al.* 1976). However, these workers showed that the effect of these acids on stearic acid was considerably less if they were unprotected in the rumen, which suggests that during feeding of whole cottonseed most of the CPFA's are probably saturated. The CPFA's can be carried into the food chain by ingestion of animal products that have been fed with them. An appreciable portion of the ingested acids is deposited in the egg yolks of hens and turkeys, and in the body fat of laying hens and dairy cattle (Anon 1973).

1. 5. 3. 2 Phytate

Phytate, the salt of phytic acid, is a cyclic compound (inosited) containing six phosphate radicals. The concentration of the phytic acid in CSM varies between 29-43 g kg^{-1} depending on different process methods (Smith 1970; Vix *et al.* 1971; Gardner *et al.* 1976).

Physiological significance of phytate lies in the fact that it readily chelates with di- and tri-valent metal ions such as calcium, magnesium, zinc, and iron to form poorly soluble compounds that are not readily absorbed from the intestines (Huisman 1989). Thus, phytate has generally been regarded as an antinutritional factor which interferes with bioavailability of minerals from plant sources (Reddy *et al.* 1982; Forbes and Erdman 1983). It has been shown that high dietary calcium accentuates the effect of phytate on zinc bioavailability. The formation of Zn-Ca-phytate complexes in the upper intestinal tract of monogastric animals is believed to be a major mechanism by which phytate reduces zinc bioavailability. The bioavailability of zinc can be best predicted by the expression: phytate-calcium/zinc molar ratio (Fordyce *et al.* 1987). Kumar and Kapoor (1983) reported that rats receiving diets containing the lowest phytate:zinc molar ratio (13.7:1) had the highest protein efficiency ratio 1.97 while rats with the highest phytate:zinc molar ratio (38.5:1) had the lowest protein efficiency ratio.

Although the ability of phytate to interfere with the availability of minerals accounts for its major antinutritional effect, phytate has also been shown to interact with basic residues of proteins. Bailey (1948) reported that phytic acid is responsible for the decreased solubility of cottonseed protein at acid pH. It is not surprising, therefore, that phytate inhibits a number of digestive enzymes such as pepsin, pancreatin, and α-amylase (Liener 1989; Savage 1989). Inhibition may also result from the chelation of calcium ions which are essential for the activity of trypsin and α-amylase, or possibly to an interaction with the substrates for these enzymes.

Phytic acid is not removed from cottonseed meal by solvent extraction or air classification (Wozenski and Woodburn 1975), but tends to concentrate in the non-storage protein isolate on fractionation (Berardi *et al.* 1969; Lawhon 1975). The phytate content can be reduced by taking advantage of the endogenous enzyme, phytase, which accompanies phytate in separate compartments of the plant tissue, or by providing an exogenous source of the enzyme from microbial sources (Liener 1987). Thus the phytate content can be greatly reduced by simply allowing aqueous suspensions of cottonseed meal to undergo autolysis under appropriate conditions of time, temperature, and pH.

1. 5. 3. 3 Aflatoxin

Aflatoxin contamination of cottonseed is uncommon, and where it does occur the level is usually very low. Nevertheless, high levels of aflatoxin B_1 are occasionally found in seeds from bolls that have been infected with *A. flavus* (Marsh *et al.* 1969). Aflatoxin, whose presence is readily detected by its bright greenish-yellow fluorescence (Marsh and Simpson 1984), tends to be carried with the protein when contaminated cottonseed is

processed into meal, concentrates and isolates (Stoloff *et al.* 1976). It is insoluble in hexane but can be extracted from cottonseed meal by polar solvents such as aqueous 2-propanol (Rayner *et al.* 1977).

The aflatoxins can be inactivated by treating the contaminated cottonseed meal with ammonia at elevated temperature and pressure (Gardner *et al.* 1971). The effect of this treatment on the aflatoxin and the protein quality of the meal have been studied (Lee and Cucullu 1978; Conkerton *et al.* 1980). Although the treatment is not approved for human use, fears that the products of ammoniation might be mutagenic have been allayed (Lawlor *et al.* 1985).

1. 5. 3. 4 Allergens

Cottonseed, in common with other oilseeds, contains a number of allergens that are capable of inducing allergic responses in hypersensitive people (Frank 1987). Most of these allergens are water-soluble and are readily isolated from solvent-extracted CSM by extraction with water (Bailey 1948). The most potent cottonseed allergen, CS-1A, comprising 13.8 g kg^{-1} of the meal, is identical to a cottonseed 2S protein as judged by gel-electrophoretic pattern, amino acid composition and immuno-cross-reactivity (Spies *et al.* 1951; Youle and Huang 1979). Another type of allergen, designated 2CS, is present in the water-insoluble globulin fraction of cottonseed. Little is known about this allergen (Frank 1987). The water soluble antigens found in cotton bract or textile mill dust implicated in the byssinosis of cotton mill workers are not present in defatted CSM or hulls.

1. 6 EFFECT OF HEAT AND SOLVENT EXTRACTION ON THE NUTRITIVE VALUE OF COTTONSEED MEAL

Table 1. 10 presents the nutrient composition of CSM is produced by the three primary processes (screw press, prepress solvent extraction and direct solvent extraction). They were derived from a comprehensive study involving more than 1,300 individual analyses (Jones 1981).

Screw pressed CSM is relatively high in residual lipid and is low in free gossypol and has a low protein quality for non-ruminant animals. The high heat and pressure conditions of screw pressing seem to decease the availability of some of the lysine (Table 1. 10). Prepress solvent meals are low in residual lipid and free gossypol and have moderate to high protein quality. The direct solvent meals have a high protein quality, have moderate residual oil, and high free gossypol. In a comparison of different processing methods (Luo *et al.* 1994), screw pressing yielded a lysine availability for

chicks of 0.53, prepressing yielded a lysine availability of 0.76 and direct solvent meal yielded a lysine availability of 0.83.

Table 1. 10 Mean nutrient composition of cottonseed meal by process (on an "as fed" basis; Sources: Smith 1970; Jones 1981)

Composition		Screw Press	Prepress Solvent	Direct Solvent
Dry matter	g kg^{-1}	914	899	904
Ash	g kg^{-1}	62	64	64
Crude fibre	g kg^{-1}	135	13 6	124
Ether extract	g kg^{-1}	37.2	5.8	15 1
Crude protein	g kg^{-1}	410	414	414
N-solubility[1]	g kg^{-1}	368	544	694
EAF lysine[2]	mg 16mg N^{-1}	23.6	30.2	34.8
Gossypol				
Free	g kg^{-1}	0.4	0.5	3.0
Bound	g kg^{-1}	9.8	10.8	7.4
Total	g kg^{-1}	10.2	11.3	10.4

1 Nitrogen soluble in 0.02 N NaOH.
2 Epsilon amino free lysine "available lysine" (Rao *et al.* 1963).

In the processed meal, the remaining free gossypol plus that which is bound, equals the total gossypol. Therefore, the total gossypol content of CSM is not affected by the process used in oil extraction. Free and total gossypol contents for different meals are given in Table 1. 10. The degree of binding is also critical due to the importance of available lysine, especially when the meal is to be fed to monogastric animals. This creates the trade off in CSM where more bound gossypol results in a lower level of the already marginal lysine. It is unknown at this time if some of the bound gossypol, is released in the gut of the animal (Martin 1990) and this is a fertile area for further research.

For ruminants, the value of dietary protein is influenced by the proportion of amino acids bypassing the rumen without being degraded to ammonia (Chalupa 1984; Preston and Leng 1987). The rumen degradabilities of CSM protein vary, resulting in different bypass characteristics. Screw press meals usually have a low rate of ruminal solubility, hence one would expect that greater amounts of dietary protein in this type of meal are presented to the intestine for digestion and assimilation by the animal. Goetsch and Owens (1985) compared the ruminal degradation of CSM protein from different commercial processing methods (screw press, SP; prepress solvent, PP and direct solvent extraction, DS). They found that the rate of *in situ* N disappearance tended to be greater for DS than for PP and SP meals. In the cow trial, ruminal degradation of supplemental N was lowest for the SP meal (Table 1. 11). Rumen degradation of CSM N did not differ significantly with processing method in the trial with steers, though trends were

similar to those found in the cow trial. In both trials, organic matter (OM)and starch digestion were decreased in the rumen and increased post-ruminally with SP as compared with PP and DS. They concluded that the processing method of CSM altered site of protein and OM digestion and that protein degradation will vary with experimental conditions.

Table 1. 11 Ruminal degradation of nitrogen (N) and digestibilities of different commercial processed cottonseed meal (CSM) fed to lactating cows and steers (Source: Goetsch and Owens 1985)

	Cottonseed meal		
	Screw press	Prepress solvent	Direct solvent
N solubility (% of N; in 0.15 N NaCl)	15	29	27
N disappearance (*in situ*)			
4 to 12 h (%/h of 4-h residue)	1.0	2.0	2.0
12 to 20 h (%/h of 12-h residue)	2.0	1.0	2.6
In the study with cows:			
Ruminal degradation of supplemental N (% of intake)	43 (57)[1]	65 (62)	65 (64)
Microbial efficiency (g microbial N/kg OM fermented)	23 (16)	19 (14)	22 (16)
Organic matter digestibility (%)			
Ruminal	35 (54)	45 (58)	43 (57)
Post-ruminal	20 (15)	13 (11)	8 (11)
Starch digestibility (%)			
Ruminal	51 (78)	67 (80)	69 (80)
Post-ruminal	28 (15)	29 (12)	45 (9)

1 Numbers presented in parentheses are results obtained from the study with steers.

Table 1. 12 presents bioavailable energy values for the various commercially available cottonseed meals. The level of oil in a meal greatly influences the energy level. As noted, screw pressed meals are relatively high in residual lipids, while prepress solvent meals are low and direct solvent meals are intermediate.

1. 7 THE EFFICIENCY OF UTILISATION OF COTTONSEED MEAL FOR ANIMAL PRODUCTION

CSM has long been a popular and economic protein concentrate for animal feeding. A relatively low cost has been the prime reason why this by-product of the cottonseed oil extraction industry has been used in feeds for many classes of animals. However, CSM has some natural limiting factors that must be considered for its safe use, particularly for nonruminant animals. Chief among these factors are protein level and quality, fibre level, gossypol and condensed tannin contents (Stern and Ziemer 1993; Terrill *et al.* 1992a;

Batterham 1992; Martin 1990). In this section, the efficiency of utilisation of CSM in the diets of different animal species is reviewed. The latest recommendations for safe levels of gossypol and CPEA's in the diets are also reviewed.

Table 1. 12 Energy values of commercially processed cottonseed meal (MJ kg^{-1}; on an "as fed" basis; Source: Jones 1981)

Species and type of meal	Energy value[1]				
	DE	ME	ME-N	NE*p*	TME
Poultry:					
Screw press			9.5		
Prepress solvent			9.0		9.4
Direct solvent			9.1		10.2
Pig:					
Screw press	11.1	10.3			
Prepress solvent	10.9	9.8			
Cattle:					
Screw press	13.3	9.8		6.8	
Prepress solvent	13.4	9.8		7.5	
Direct solvent	13.6	10.4		6.1	
Sheep:					
Screw press	13.9	10.3		7.4	
Prepress solvent	14.4	11.1		7.6	
Direct solvent	14.3	11.1		7.2	

1 DE, digestible energy; ME, metabolizable Energy; ME-N, metabolizable Energy corrected for nitrogen retention; NE*p*, net energy for production; TME, true metabolizable Energy by the method of Sibbald (1976).

1. 7. 1 Cottonseed Meal in Pig Diets

CSM can replace a portion of the soya bean meal in pig diets and maintain equal performance, if the nutrient content and presence of free gossypol in CSM are considered in diet formulation (Tanksley and Knabe 1981). Too many times the popular conception of CSM is based on feeding trials conducted many years ago in which CSM, often of poor quality, was the only source of supplemental protein. Many early workers, however, have clearly demonstrated that excellent pig performance, often superior to soya bean meal alone, has been obtained when limited amounts of CSM were fed in combination with other sources of high quality protein in practical growing-finishing diets (Sewell *et al.* 1955; Haines *et al.* 1955; Hale and Lyman 1957; Hale and Lyman 1961; Tanksley and Lyman 1966, Hintz and Heitman 1967; Tanksley 1969).

The protein quality of CSM for pigs is limited by low levels of several essential amino acids, particularly lysine. Protein quality is further lowered if the CSM is processed at high temperatures, which promotes a reaction between free gossypol and free amino groups in the protein to form an indigestible complex; lysine, because of its

free epsilon-amino group, is primarily affected. The lower digestibility (Tanksley *et al.* 1981) and availability (Batterham *et al.* 1990) of lysine in processed CSM, compared with soya bean meal, has been reported. Supplementation of cereal grain-CSM based grower-finisher pig diets with synthetic lysine has consistently improved pig performance (Aguirre *et al.* 1960; Bell and Larsen 1963; Noland *et al.* 1968; LaRue *et al.* 1985, 1987; Ikurior and Fetuga 1988 and Batterham *et al.* 1990). Tanksley and Knabe (1981) have shown that lysine was the least digestible essential amino acid in processed CSM (Table 1. 13). They found that the digestibilities of the essential amino acids isoleucine, leucine, methionine, threonine, tryptophan and valine were also lower when compared with soya bean meal. Batterham (1992) discussed the availability and ileal digestibility of some amino acids in CSM for growing pigs and summarised that for lysine, threonine, methionine and tryptophan, heating processed CSM induced changes which depressed ileal digestibility slightly but appeared to result in a substantial proportion of these amino acids being absorbed in inefficiently utilised form(s). In contrast, the branched chain amino acids, isoleucine, leucine and valine, appeared less susceptible to the effects of heat. Thus, for these amino acids, reduced ileal digestibility appeared to be the main cause of reduced availability.

Table 1. 13 Apparent digestibility values for N and essential amino acids in cottonseed meals and soya bean meal as determined at the terminal ileum of growing-finishing pigs (Source: Tanksley and Knabe 1981)

	Cottonseed meal			Soya bean
	Direct solvent	Screw press	Glandless	meal
Nitrogen	0.73	0.75	0.86	0.81
Lysine	0.62	0.64	0.87	0.86
Arginine	0.88	0.90	0.96	0.90
Histidine	0.80	0.81	0.92	0.87
Isoleucine	0.68	0.70	0.84	0.82
Leucine	0.70	0.73	0.85	0.81
Methionine	0.71	0.66	0.83	.088
Phenylalanine	0.81	0.82	0.90	0.85
Threonine	0.62	0.65	0.79	0.75
Tryptophan	0.74	0.68	0.83	0.80
Valine	0.71	0.71	0.85	0.80

The digestibility of lysine in a laboratory-processed glandless CSM (0.87) equalled that of soya bean meal, and digestibilities of other essential amino acids were similar or higher to soya bean meal. With supplemental lysine, glandless CSM can replace about one-half of the soya bean meal protein in corn-based diets without reducing pig performance (LaRue *et al.* 1987).

Commercial CSM contains 120-130 g crude fibre kg^{-1} DM, which lowers its digestible energy content for pigs. Husby and Kroening (1971) found an inverse relationship between crude fibre content and digestible energy values for screw press and prepress solvent CSM. Higher dietary crude fibre content on CSM in pig diets may slightly depress feed efficiency, but the effect should be minimal. Substitution of CSM for soya bean meal in a grower diet will increase dietary fibre content by only 1% (Tanksley and Knabe 1981). LaRue *et al.* (1985) compared energy values in glandless CSM and soya bean meal using growing and finishing swine and found that digestible and metabolizable energy for glandless CSM were significantly ($P<0.01$) lower than those for soya bean meal, reflecting the high on fibre content. Digestibility of gross energy was lower at both the end of the small intestine and over the total tract.

Extensive research has been conducted to determine the level of dietary free gossypol which adversely affects pig performance. Although several factors (e.g. age, protein and lysine levels in the diets) affect the tolerance of pigs to free gossypol, feed consumption and daily gains are decreased and toxicity symptoms usually occur when the free gossypol level approaches 100 ppm in the complete diet (Holley *et al.* 1955; Hale and Lyman 1957; Clawson *et al.* 1961; Kornegay *et al.* 1961). Complete pig diets containing up to 100 ppm of free gossypol can be fed without fear of gossypol toxicity. Going above 100 ppm in the diet is not necessarily a problem, since extensive research has shown that iron salts are effective in blocking the toxic effects of dietary gossypol (Withers and Carruth 1917; Robison 1934; Clawson *et al.* 1967; Knabe *et al.* 1979). It is thought that the iron binds to free gossypol (NCPA 1966). The recommendation is a 1:1 weight ratio of iron salts to free gossypol in diet containing more than 100 ppm (as-fed basis) of free gossypol (Tanksley and Knabe 1981). However, the safe limit even with the inclusion of iron is 400 ppm of free gossypol in the complete diet (NCPA 1970).

With present methods of processing and without the addition of synthetic lysine, screw press meals have limited usefulness in pig rations. Prepress solvent and direct solvent meals are preferred for pig diets because of their better protein quality. For maximum gain and feed efficiency, CSM must be fed in combination with other protein feedstuffs such as soya bean meal and fish meal, which are rich in the essential amino acids. CSM is best utilised in grower-finisher pig diets and sow diets (Tanksley and Knabe 1981). CSM can also be effectively utilised in limited amounts in gestation and lactation diets (Haught *et al.* 1977). It can be concluded that good pig performance can be obtained on diets containing 50% supplemental protein from CSM if the remaining 50% supplemental protein is provided by other high quality sources, such as soya bean or fish meal or a combination of both.

1. 7. 2 Cottonseed Meal in Poultry Diets

For many years the use of CSM in poultry diets has been limited by problems associated with the presence of free gossypol in the meal. However, research has also indicated that at least some of the poor results associated with feeding CSM could be attributed to a lysine deficiency and/or CPFA's. Recognition of these factors, coupled with improved processing techniques, has made possible the utilisation of a greater percentage of CSM in poultry feeds.

The major hazard associated with the presence of gossypol in poultry feeds is the formation of an iron-gossypol complex which may occur in the digestive tract, the bloodstream, or in the yolk of the egg (Waldroup 1981). The laying hen is the most sensitive to the level of gossypol, as yolk discoloration is observed at free gossypol levels far below those needed to inhibit growth or cause mortality. Eggs that are even marginally discoloured must be considered as a loss and may result in an adverse consumer response. Since the discoloration may develop after the eggs have left the farm, it is extremely important that the possibility of gossypol discoloration be avoided. Broilers or turkeys that are fed marginal excesses of free gossypol may have a reduction in weight gain but are still able to be sold. The free gossypol levels determined in the complete diets as safe for poultry are 50 ppm for layers and 100-150 ppm for broilers (NCPA 1970; Waldroup 1981). The recommendation is 4 ppm additional iron for each 1 ppm of free gossypol for layers and a 1-2:1 ratio of additional iron to gossypol is recommended for broilers. Even with the inclusion of iron salts, it is suggested that the maximum gossypol levels in the diets be 150 ppm for layers and 400 ppm for broilers on an as-fed basis (Waldroup 1981; Martin 1990).

The major amino acid deficiency in CSM is lysine. Not only is the total amount of this amino acid low (about 40.9 g kg^{-1} of the dietary protein, as compared to around 63.1 g kg^{-1} for soya bean meal), but it is also subject to the formation of indigestible complexes with gossypol or carbohydrates during processing. Studies conducted by Packham *et al.* (1973) assayed different samples of screw press CSM for lysine availability by chemical tests. They found that the proportion of lysine chemically unavailable was 15% for a mildly heat-treated meal and 38% for an overheated sample. As the level of CSM in the diet is increased, attention must be given to the quantity of other essential amino acids. Studies with both conventional and glandless CSM fed to growing chicks (Fisher *et al.* 1971) suggested that threonine, leucine and isoleucine became limiting after lysine and methionine. Since it is not commercially feasible to add these amino acids (except threonine) to diets, their needs must be met from the intact protein sources in the diet.

CSM, or more specifically the residual oil, may contain quantities of CPFA's. The presence of these fatty acids in CSM may result in the development of a pink discoloration of egg albumen. This pink discoloration is formed when yolk iron diffuses into the albumen and forms a chelate with conalbumen (Phelps *et al.* 1965). The CPFA's cause an increase in permeability of the vitelline membrane of the yolk. Therefore, CSM fed to laying hens should be selected to be as low as possible in residual lipids.

Laying hen diets represent the most critical area of CSM use, due to the potential market loss of eggs from gossypol or CPFA discoloration. However, much of the CSM used in poultry feeds finds its way into layer diets. Studies by Waldroup and Goodner (1973) confirmed that feeding levels up to 50 ppm free gossypol in the diet to laying hens, with or without the addition of supplemental iron salts, had no adverse effects on rate of egg production, egg weights and feed utilisation. Eggs stored up to 12 weeks were examined, and no indication of yolk or albumen discoloration was observed. They also reported that no adverse effects on rate of egg production were observed at free gossypol levels up to 200 ppm with supplemental iron at a 4:1 ratio. Production was reduced at 400 ppm gossypol but did not differ significantly from that of hens fed the control diet. Studies were conducted (Waldroup *et al.* 1976) to determine the effect of CSM (up to 15%) in diets containing either corn or milo as the sole grain, and results indicated that there were no differences in rate of egg production, egg weight or interior egg quality related to either the level of CSM or the grain source used. Roberson (1970) conducted a study over two laying periods in which 50 or 100% of the soya bean meal in a layer diet was replaced with glandless CSM. The results showed that the replacement had no adverse effects on rate of egg production, feed utilisation, egg weight, body weight gain or mortality. However, significant increases in yolk discoloration and pink whites were observed, with the incidence increasing as the length of storage time increased. Quisenberry and Delfino (1971) conducted two studies with laying hens in which glandless CSM completely replaced the soya bean meal. They found that rates of egg production and feed utilisation were significantly better for hens on glandless CSM diets, and adverse effects on yolk or albumen discoloration were not observed after 90 days storage of eggs.

Studies conducted by Watts (1970) demonstrated the importance of the source of CSM used in broiler diets. In this study, different sources of CSM processed by screw press, prepress solvent and direct solvent techniques were incorporated into broiler diets at 75 and 150 g kg^{-1}, and free lysine was added as needed to meet the needs for this amino acid. Iron was added at an 0.85:1 ratio of iron: free gossypol. The results of this study showed that free gossypol levels up to 180 ppm resulted in no adverse effects on growth rate and feed utilisation by chicks. Packham *et al.* (1973), and Packham and Payne (1973) reported that broiler diets supplemented with CSM did not support

performance equal to that obtained on diets based on soya bean meal, even though fortified with methionine and lysine. However, the free gossypol concentrations in these diets were as high as 230 ppm. In broiler studies conducted by Waldroup *et al.* (1968), up to 100% of the soya bean meal was replaced by glandless CSM with no adverse effects on growth or feed utilisation when the diets were adequately supplemented with lysine. Waldroup *et al.* (1980) determined the minimum levels of supplemental lysine needed when glandless CSM was used to replace various levels of soya bean meal. Diets containing glandless CSM up to 260 g kg^{-1} (75% replacement of soya bean meal) required no additional lysine supplementation to support performance equal to that attained on the soya bean meal control diet.

CSM can be used effectively to provide a significant portion of the protein in the diet of layers, broilers and growing pullets. CSM has been very valuable in many areas in helping to lower the cost of poultry diets, but care must be given to their usage. For layers and broilers, it is concluded that up to 150 g kg^{-1} of CSM (410 g crude protein kg^{-1} DM) fortified with ferrous sulphate can be included in the diets. This comprises approximately 30-40% of the total supplementary protein.

A key factor which will determine whether increased amounts of CSM are utilised in poultry feeds will be the decision of the cottonseed processor to produce a quality product intended for monogastric usage. Prepress CSM can be produced which are low in free gossypol and residual oils, and these contain almost 50% protein with minimal processing damage, and are much lower in fibre and consequently higher in energy than the majority of the CSM presently produced. Such meals would allow the poultry industry to utilise CSM in their diets with greater confidence and could contribute to the economical production of poultry in many areas of the world.

1. 7. 3 Cottonseed Meal in Ruminant Diets

The amount of undegraded digestible protein reaching the small intestine of ruminant animals may limit performance of high producing animals. Current concepts of protein use by ruminants, particularly for lactating dairy cows, include metabolizable protein or amino acids available for absorption in the small intestine (Coppock *et al.* 1987) as: the sum of metabolizable protein and amino acids present and available in microbial protein plus those in dietary protein which has not been heat-damaged or degraded by passage through the rumen (Satter and Roffler 1975). It is now accepted that microbial protein does not provide enough amino acids for high milk production (Chalupa and Sniffen 1994), although a rumen ammonia concentration high enough to promote maximal microbial growth (Satter and Roffler 1975) is considered essential. The undegraded feed protein should have an amino acid composition which complements the microbial

protein, and the sum should match requirements for milk production or tissue growth for the young calf. Feed processing methods have been suggested to influence the extent of ruminal escape of protein in the feed (Preston and Leng 1987).

A large part of the protein in CSM can be protected from microbial degradation in the rumen by the heat applied during processing. The degree of protection of protein in CSM has been shown to vary with the methods of processing of the meal (Goetsh and Owens, 1985). Heat treatment of CSM has been used to reduce ruminal degradation and to increase the amount of CSM protein digested in the small intestine (Broderick and Craig 1980). The same workers also showed a large range in the degradation of protein in commercial samples of CSM, suggesting that during processing the meal was exposed to a wide range of temperature and moisture. Pena *et al.* (1983) showed that extruding or roasting whole cottonseed decreased rumen ammonia N concentration and increased crude protein flow at the duodenum and increased the apparent absorption of both indispensable and dispensable amino acids from the small intestine.

CSM is very useful as a supplement for ruminants fed low quality roughage diets (Hennessy and Murison 1982; Lee *et al.* 1987), and low quality forage based diets (Judkins *et al.* 1991). Hennessy and Williamson (1993) compared fresh brewer's yeast slurry and CSM as protein supplements for beef cattle given native pasture. They found that when CSM (1 kg d^{-1}) was added to the hay diet, there was a significant improvement in live weight gain and feed conversion efficiency compared with adding 4.4 kg d^{-1} of yeast slurry to the diet. Cottonseed cake as a supplement to replace soya bean meal for feeding fattening sheep has also been reported (Kandyis *et al.* 1992).

Sanchez and Claypool (1983) compared CSM to canola meal and soya bean meal as protein supplements for lactating cows fed isocaloric and isonitrogenous complete rations. Milk yields for the CSM supplement were relatively high, but neither actual yields nor fat corrected milk (FCM) yields differed significantly among meals, nor did percentages or yields of major milk components. Total rumen volatile fatty acid content for CSM was lower than that for other meals. In a comparison of CSM with peanut meal and soya bean meal in diets with 135 or 164 g crude protein kg^{-1}, Van Horn *et al.* (1979) found that cows on the CSM diet had lower feed intakes and milk yields than animals on other meals of 135 g kg^{-1} level, but at the 164 g kg^{-1} level results were similar. Organic matter digestibility was depressed in CSM diets (0.63) compared to peanut meal diets and soya bean meal diets (0.68 for both diets). These results indicated that as a protein supplement for lactating cows CSM has a similar nutritive value to soya bean and other meals.

In a comparison of canola meal, CSM and soya bean meal as protein supplements for calves, 8 weeks preweaning plus 8 weeks post-weaning, Claypool *et al.* (1985) found

no significant differences in gains, packed cell volumes or thyroxine concentrations in blood. However, Randel *et al.* (1992) suggested that producers should avoid feeding cottonseed products containing free gossypol to pre-ruminant animals.

There have been occasional press reports and scientific reports (Rogers *et al.* 1975; Holmberg *et al.* 1988; Randel *et al.* 1992) of what was assumed to be the practical impact of free gossypol on pre-ruminant calves. When cottonseed products are fed to young calves with a functionally undeveloped rumen, gossypol toxicity is a potential problem (Leighton *et al.* 1953; Hollon *et al.* 1958; Ensminger and Olentine 1978; Orgad-Klopfer and Adler 1986; Holmberg *et al.* 1988; Kerr 1989). Clinical signs and postmortem findings similar to those observed in pigs have also been reported by these authors. Based on the data currently available, the use of CSM in the diet of pre-ruminant calves or lambs requires understanding of what time period encompasses the pre-ruminant period. It appears that a safe level, for pre-ruminants may be maximum of 100 ppm in the total diet (Martin 1990). This is based primarily on the observation by Hollon *et al.* (1958) as well as on the safe levels for growing pigs.

Today's high milk production levels have caused some new concern with regard to gossypol toxicity in ruminants. There is a concern that high levels of CSM feeding could tax the rumen's gossypol detoxification ability. Lindsey *et al.* (1980) found gossypol in the plasma and livers of dairy cows fed a diet containing 450 g direct solvent and screwpress processed CSM kg^{-1}, indicating that the consumption of 242 g free gossypol per head per day for these high-producing Holsteins was too much. However, the study did not reveal how much gossypol could be safely handled by the high producing dairy cow. If too much CSM and whole cottonseed is fed, it could cause the gossypol level in the rumen to reach or exceed the detoxification limit. The problem could be exaggerated when whole cottonseed is fed in addition to a concentrate that contains CSM (Randel *et al.* 1992). The usual safe recommendations for high-producing cows vary from 2.3-3.6 kg of whole cottonseed per head per day (Bath 1976; Jimenez 1980; Jimenez *et al.* 1989; Coppock 1984; Martin 1990).

Lusas and Jividen (1987) reported that glandless CSM can be used as a milk replacer for calves whose rumen is still in the non-functional state. Research has shown that, at equivalent fibre levels, the metabolizable energy of solvent extracted glandless CSM is approximately 20% higher than that for glanded prepress solvent extracted meal (Wilcke 1978). Harper (1967) reported that glandless CSM contained approximately 17% more lysine than glanded meals produced in the same oil mill.

CSM is a very good protein supplement for ruminants fed low-quality roughage or forage based diets. CSM as a protein supplement for lactating cows has a similar nutritive value to soya bean and other meals, but high levels of CSM feeding could tax

the rumen's gossypol detoxification ability. Cottonseed products containing free gossypol should not be fed to pre-ruminant calves and lambs.

1. 8 FEEDING VALUE OF WHOLE COTTONSEED AND COTTONSEED HULLS TO RUMINANTS

1. 8. 1 Whole Cottonseed

Whole cottonseed (WCS) is a unique feedstuff with high amounts of energy, protein and fibre. Oil (ether extract), crude protein and NDF composition of whole cottonseed are 210, 240 and 390 g kg^{-1} DM (Table 2), respectively. In addition to these factors, effectiveness of the NDF in WCS was found to be worth 1.23 times the NDF in alfalfa (Clark and Armentano 1992). The diet of the high-producing dairy cow can be deficient in energy and fibre and WCS is an important feedstuff that can contribute both of these nutrients.

Utilisation of WCS in diets for lactating cows often increases milk yield and milk fat test, but decreases milk protein content (Coppock and Wilks 1991). Coppock *et al.* (1987) summarised 18 studies with WCS and found no significant reduction in DM intake when WCS was included at up 25% of the ration, which suggests that in most trials an increase in net energy for lactation (NE_l) probably occurred. DM intake was significantly depressed by WCS, when it was included at 30% of the diet (Chik *et al.* 1985). These authors also noted that milk production responses were variable, usually small, but positive. The effect of the inclusion of WCS was to increase milk fat percentage (8 out of 13 trials) and frequently to increase the yield of FCM. About half the studies showed depressions in milk protein percentage with one-half of these being significantly lower than the controls. No consistent effects were seen for the digestibility of DM, nitrogen, crude fibre, acid detergent fibre, calcium, phosphorus or magnesium. The digestibility of the ether extract usually increased with inclusion of WCS.

Probably there are several mechanisms combining to produce the effect of WCS increasing milk fat content. Cotton fibre has a crude fibre digestibility of 0.91 for sheep (NRC 1971). The digestion of lint (cellulose) yielded acetic acid of a proportion that would increase milk fat synthesis (Bauman and Davis 1974). Crude fibre in whole linted cottonseed has a digestibility of 0.58 (NRC 1971) to 0.64 (Morrison 1959). As emphasised by Palmquist and Jenkins (1980), addition of supplemental fat allows a reduction in dietary starch and an increase in dietary fibre while increasing energy density. This is especially true when the fat (oil) carrier is the high fibre ingredient, WCS. The disappearance of 99% of whole linted seeds during intestinal passage (Coppock *et al.* 1985a) suggests their stratification in the rumen with corresponding

regurgitation and mastication. Smith *et al.* (1981) further showed that although synthesis of milk fatty acids C_6-C_{12} was depressed as WCS was increased in the diet, the yields of stearic and total oleic acids doubled on a 250 g kg^{-1} WCS diet compared to a basal diet.

The depression in protein percentage of milk, observed with WCS feeding is not specific, but occurs with other forms of supplemental fat, especially protected lipid (Palmquist and Jenkins 1980). It has been shown to occur in the casein fraction with coconut oil by Storry *et al.* (1974), with protected tallow by Dunkley *et al.* (1977), and with WCS by DePeters and Bath (1985). The mechanisms have yet to be defined. Palmquist and Moser (1981) suggested that supplemental fat feeding may induce insulin resistance and cause a reduced transfer of amino acids into the mammary gland and hence reduce milk protein synthesis.

Addition of fat to ruminant diets has often depressed fibre digestibility (Palmquist and Jenkins 1980). Devendra and Lewis (1974) suggest that four mechanisms are operative in the rumen to explain this depression. The feeding of cottonseed oil depressed fibre digestion in a high-roughage ration but not in a low-roughage ration, when compared to a control and WCS ration (Moody and Barnes 1978). Palmquist and Jenkins (1980) note that most data suggest an inhibitory effect on microbial activity. They also concluded that feeding supplemental calcium, especially calcium chloride (Jenkins and Palmquist 1982; 1984) was effective in preventing the depression in fibre digestibility often seen with added fats or oils. Studies summarised by Coppock *et al.* (1987) did not suggest any depression in fibre digestibility when WCS was included at up to 300 g kg^{-1} of the diet.

It should be emphasised that the impact of feeding WCS on animal performance appears to be influenced by forage type in the diet (Harris 1991). Five studies summarised by Staples *et al.* (1991) showed that when corn silage was the primary forage source, milk production increased and milk fat decreased with the addition of 100-150 g WCS kg^{-1} to the diet. In contrast, Harris (1991) summarised five studies that showed a different effect when WCS was added to diets where the primary forage source was alfalfa. WCS addition at 100-300 g kg^{-1} had little effect on milk yield with alfalfa in the diet, but consistently increased milk fat percentage.

Horner *et al.* (1988a) evaluated effects on ruminal fermentation of including 0 or 150 g kg^{-1} WCS in diets fed to cannulated heifers. Effects of WCS (up to 30% of the total dietary DM) on *in vitro* ruminal fermentation increased ruminal pH and ammonia concentration but lowered microbial protein (Horner *et al.* 1988b). Acetate concentration was greatest with diets of 150 and 300 g WCS kg^{-1}, but propionate and total VFA concentrations were lowered by increasing WCS from 0 to 300 g kg^{-1}.

Protozoal numbers were reduced. Reduction of rumen protozoal numbers with WCS feeding has been reported by others (Mohamed *et al.* 1988; Kajikawa *et al.* 1991).

WCS is an important feed supplement for dairy cows, particularly for supplementing energy and fibre for the high-producing cow. However, if too much WCS is fed to animals, it could cause gossypol toxicity. The safe recommendation for WCS fed to dairy cows is to feed no more than 3.6 kg of WCS per head per day.

1. 8. 2 Cottonseed Hulls

Cottonseed hulls (CSH) are a by-product of cottonseed processing. CSH consists primarily of the outer covering of cottonseed with lint fibres clinging to the hulls (Harris 1991). Because of the extremely high NDF content (900 g kg^{-1} DM; NRC 1989) and effective ADF (660 g kg^{-1} DM; Stern and Ziemer 1993) in CSH, they are commonly used in cattle diets to supply fibre.

Determination of digestibility for diets with CSH gives results that are largely predictable from the digestibilities of the components: CSH substituted for alfalfa decreased diet digestibility (Brown *et al.* 1977) and CSH substituted for ground corrugated boxes increased diet digestibility (Peavy *et al.* 1980). Total tract digestibilities of DM, OM, NDF and ADF were 0.50 or less for sheep fed CSH diets, compared with more than 0.70 for sheep fed corn fibre and soya bean hulls (Hsu *et al.* 1987). Tuncer *et al.* (1992) reported that the *in situ* DM, NDF and ADF digestibilitiesof CSH in the rumen were 0.35, 0.34 and 0.30, respectively, and that urea, ammonia, NaOH and $CaOH_2$ treatments did not improve digestibility. In low-fibre diets, CSH may have a special value because of a stimulatory effect on feed intake, but not through improvement in digestibility (Coppock *et al.* 1987).

An early study in USA (Lush *et al.* 1933) showed that CSH had a relative roughage value for milk production greater than hill pasture hay and bermudagrass hay, nearly equal to bermudagrass, and inferior to mixed clover hay. CSH are palatable, causing only a slight depression in intake, compared to alfalfa hay (Irwin *et al.* 1981). There is no effect on intake when CSH is substituted for corn and barley (Nik-Khah and Hassanyn 1981). Harris *et al.* (1983) compared corn silage and CSH in several experiments. They found that cows in mid-lactation fed CSH had a higher DM intake than cows fed corn silage; however, there were no differences in milk yield, milk fat and milk protein. In another trial, they observed no differences in DM intake, milk yield and composition when cows were fed diets with 35 g kg^{-1} CSH or 25 g kg^{-1} sunflower hulls with 10 g kg^{-1} CSH. Van Horn *et al.* (1984) noted that cows fed CSH diets had greater DM intake, milk yield and milk protein and lower milk fat than cows on sunflower hull diets. Morales *et al.* (1989) showed that cows in mid to late lactation fed 30 g kg^{-1} CSH

had greater milk yield and protein yield but less milk fat and yield, compared with cows fed 30 g kg^{-1} alfalfa hay alone. In most studies (Harris *et al.* 1983; Van Horn *et al.* 1984), milk yields follow energy intake (also CSH intake) with milk fat percentage being highly variable.

There is relatively little information on the effect of CSH on ruminal fermentation, compared with other fibrous by-products. Hsu *et al.* (1987) evaluated the potential of corn fibre, CSH, oat hulls and soya bean hulls as roughage sources for ruminants. *In situ* rate and extent of ruminal DM disappearance indicated that corn fibre and soya bean hulls were more fermentable in the rumen, compared with oat hulls and CSH. Ruminal pH and molar propionate were greater while total VFA concentration and molar acetate were lowest for CSH, compared with the other by-products.

1. 9 CONCLUSIONS AND SOME FUTURE RESEARCH NEEDS

1. 9. 1 Cottonseed and cottonseed products have been widely investigated in the past half century. During this period, processes have been developed to prepare cottonseed oil, CSM and other associated products. Currently, there are three major methods of cottonseed oil extraction in use, namely screw pressing, prepress solvent extraction and direct solvent extraction. These methods use different temperatures, pressures and cooking times. High temperature and pressure favours the formation of stable-bonds between gossypol and other molecules. At the same time, however, damage to the meal protein has to be prevented. Screw press meals are relatively high in residual oil, low in free gossypol and have a low protein quality for non-ruminants, but degradation in the rumen is lower, resulting in a greater portion of the dietary protein being presented to the small intestine for digestion. Prepress solvent meals are low in residual oil and free gossypol and are of moderate to high protein quality. The direct solvent meals are high in protein quality, moderate in residual lipids and high in free gossypol.

1. 9. 2 Gossypol, the polyphenolic pigment, can be seen as black specks in the stems, leaves, green bolls, and in the seed of cotton. Gossypol in seed occurs in the free form, but as a result of processing, both free and bound gossypol are present in meal. Free gossypol can be toxic to animals if fed in large amounts, particularly to monogastric animals. Heat of processing is used to bind free gossypol, with the aim of providing concentrations of free gossypol not greater than 0.4 g kg^{-1} (400 ppm) in CSM.

1. 9. 3 CSM can be fed to monogastric animals and maintain equal performance, but the proportions of CSM in the diets have to be limited. This is attributable to its

contents of ANFs, such as gossypol, CPFA's and its lower level of lysine compared with soya bean meal. CSM is first-limiting in lysine, and reduced availability of lysine occurs during commercial processing. The loss of available lysine in CSM is due partly to its reaction with gossypol, but other compounds are also involved. Under present methods of processing, screw press meals have limited usefulness in monogastric rations. Prepress solvent and direct solvent meals are preferred for monogastric diets. For maximum live weight gain and feed efficiency, CSM must be fed in combination with other high quality protein feedstuffs such as soya bean meal and fish meal.

1. 9. 4 CSM is very useful as a protein supplement for ruminants fed low quality roughage and low quality forage based diets. CSM as a protein supplement for lactating cows has a similar nutritive value to soya bean and other meals. CSM produced by methods involving high temperature and pressure (i.e. screw pressing) reduces ruminal degradation and increases the amount of protein digested in the small intestine. WCS is a unique feedstuff for dairy cows. Supplementary WCS, as an energy and fibre supplement for the high-producing dairy cow, often increases milk yield and milk fat content, but decreases milk protein content. However, high levels of WCS feeding could tax the rumen's gossypol detoxification ability. CSH have a high NDF content and are commonly used in cattle diets to supply fibre (such as in feedlots), but the DM and NDF digestibilities of CSH are very low. Cottonseed products containing free gossypol should not be fed to pre-ruminant calves and lambs.

1. 9. 5 Condensed tannins, as a further group of secondary compounds, have been detected in commercially produced CSM (mainly in bound forms), as a result of recent developments in the butanol-HCl analytical procedure, which can separate total CT into extractable, protein-bound and fibre-bound fractions. CT are polyphenolic compounds, and have different structures in different plants. Plants first evolved CT production as a defence against attack by pathogenic bacteria and fungi, which then further evolved as a defence against being eaten by insects and herbivores and to adapt themselves to a given environment.

1. 9. 6 CT react with protein and carbohydrate, but react more strongly with protein than with carbohydrate. The reactions between CT and protein are influenced by pH, structure and molecular sizes of CT and protein properties. The reactions are strong at pH 3.5-7.0, but when pH is <3.0 and >8.0, the CT-protein complexes dissociate and protein is released from the complexes. Reactivity increases with increasing polymerisation of the CT. Proteins with open, loose conformations, high

molecular weights and high contents of proline and other hydrophobic amino acids have a high affinity for CT.

1. 9. 7 With monogastric animals, the presence of extractable CT in the diet has been shown to reduce the efficiency of protein digestion. Extractable CT depresses apparent amino acid digestibility due to a direct binding of CT to dietary proteins, and/or a reduced activity of protein-degrading enzymes, and/or increased secretion of endogenous proteins. However, the effects of bound CT upon protein digestion in the small intestine are unknown. Therefore, the effect of bound CT in CSM upon amino acid digestion and absorption in the small intestine needs to be studied.

1. 9. 8 With ruminants fed forages, low concentrations of extractable CT (20-40 g kg^{-1} DM) improve the efficiency of protein digestion and increase amino acid absorption from the small intestine. However, high concentrations of extractable CT depress feed intake, rumen fibre digestion and apparent amino acid digestibility in the small intestine. Studies are necessary to investigate the effects of bound CT in CSM upon solubility and degradability of both protein and fibre in the rumen.

1. 9. 9 Different manufacturing conditions during processing may be needed to produce CSM that has optimum nutritive value for ruminant and monogastric animals. For CSM produced as a protein supplement for monogastric animals high temperatures and pressures during processing should be avoided. CSM produced for ruminants should use controlled heat to reduce the rumen degradation of protein. Optimum processing conditions for producing the best quality CSM for each species needs to be precisely defined and any interaction between heat (including the amount of heat required), the concentration of CT required and any interaction between CT and heat need to be established. Research is needed in these areas. The research reported in this thesis concentrated upon the effect of CT in CSM for both ruminant and monogastric livestock, with particular reference to effects upon protein digestion.

1. 10 METHODOLOGY FOR STUDYING PROTEIN DIGESTION

1.10. 1 Measurement of Amino Acid Digestibility in Monogastric Animals

The commonly used procedure for determining amino acid digestibility in pigs has been the faecal index method (Kuiken and Lyman 1948). While the overall apparent digestibility measurement is not technically difficult, there are basic objections to this approach because of the presence of undigested and unabsorbed endogenous protein in the faeces, and possible microbial alterations of undigested and unabsorbed endogenous and exogenous N residues, in the large intestine. As a result, the amino acid composition of faeces in pigs fed diets that differ widely in amino acid composition and digestibility is rather similar (Mason *et al.* 1976).

From many studies, involving the determination of ileal and faecal N and amino acid digestibilities in pigs, some general findings have emerged, indicating the superior predictive accuracy of ileal digestibility values. Zebrowska (1978) highlighted the significance of differences between faecal and ileal estimates of the apparent digestibility of N and amino acids in feedstuffs used in practical dietary formulation. Some studies indicate close correlation between the apparent ileal digestibility of amino acids and animal performance. Moreover, apparent ileal digestibility coefficients have been shown to be sensitive in detecting small differences in protein digestibility due to the processing of foods (Sauer and Ozimek 1986; Knabe *et al.* 1989) compared with faecal estimates. Tanksley and Knabe (1984) concluded that ileal digestibility values offer great potential for increasing the precision of diet formulation for the growing pig. The traditional faecal measurement of amino acid digestibility is inadequate, and the ileal measurement should be used to determine amino acid digestibility.

Considerable work has been done with ileal digesta collection methods in pigs, such as the ileo-ileo and ileo-caecal re-entrant cannulation procedures, ileo-rectal anastomosis, nylon bag technique, post-valvular ileo-colic fistulation or ileo-colic post valve procedure and the postvalve T-caesium technique. These methods mainly involve the surgical implantation of cannulas. The use of cannulated pigs for the routine determination of amino acid digestibility is costly, labour intensive and time consuming. Although these methods may be appropriate for research purposes, they are unlikely to be appropriate for routine use in feed evaluation.

The method used for the assessment of ileal amino acid digestibility in the studies reported in this thesis involved collecting digesta from the ileum under anaesthesia before sacrifice of the animal (Moughan and Smith 1987; van Barneveld *et al.* 1991). Digestibility was measured with Cr_2O_3 as an indigestible marker given with

the test feedstuff. This method involves minimal disruption of normal digestive function. Digestibility data obtained using this technique coupled with a frequent feeding regime were not necessarily any more variable than those obtained from cannulated animals (Moughan 1991). In using this technique there is shedding of mucosal cells into the intestinal lumen at death. Therefore digesta samples have to be obtained by avoiding mucosal cells.

Accepting that amino acid digestibility should be based on measurement made at the terminal ileum of monogastric animals, it needs to be recognised that ileal digesta are derived both from dietary and endogenous sources. Endogenous amino acid loss is used to correct apparent digestibility coefficients to true values. True amino acid digestibility has the advantage over apparent digestibility in that it is a fundamental property of a feed ingredient regardless of the dietary conditions under which the ingredient is fed. For a given amino acid, apparent digestibility increases exponentially with increasing ingested feed because endogenous excretion, as a percent of total excretion, decreases proportionally (Sauer *et al.* 1980; Furuya and Kaji 1989; Keith and Bell 1991). Using true rather than apparent digestibility allows raw materials to be accurately compared, even if they are ingested in different quantities.

The argument as to whether apparent or true digestibility values are preferred for practical dietary formulation is inextricably linked to the approaches adopted in estimating amino acid requirement for growth. In the formulation of diets for pigs, it is assumed that the supply of digestible amino acid in a mixture of feedstuffs is equal to the sum of the supply based on the digestibility values determined for single ingredients. For feedstuffs with a lower level in one amino acid, their apparent ileal digestibility would be reduced by the influence of the endogenous ileal contribution (Furuya and Kaji 1989). As true ileal amino acid digestibility is corrected for the endogenous ileal amino acid, true rather than apparent digestibility values would be expected to be more additive (Taverner *et al.* 1981; Furuya and Kaji 1991). However, the difficulty of accurately determining endogenous excretion has prompted many researchers to recommend the use of apparent amino acid digestibility values for the formulation of diets (Austic 1983; Sauer *et al.* 1983).

Various approaches to the estimation of endogenous amino acids have been employed, such as the protein-free method, regression method, homoarginine procedure, radioactive isotope or tracer techniques and peptide alimentation method. The peptide alimentation method used in this thesis allowed investigation of the effect of dietary CT on endo-genous ileal amino acid flow in monogastric animals. In this method the animal is fed a semi-synthetic diet containing enzymically hydrolysed

casein (EHC) as its sole nitrogen source. Ileal digesta are collected and the nitrogenous fraction separated physically using large volume disposable ultrafiltration devices. The high molecular weight (MW>10,000 Da) fraction resulting from the ultrafiltration provides a measure of endogenous amino acid flow. If some of the dietary amino acids and small peptides are not absorbed, they will be removed in the low molecular weight fraction. In addition to the unabsorbed dietary amino acids and peptides, the low molecular weight fraction will contain some non-protein N, and endogenous free amino acids and small peptides. The latter if present, are expected to be at a low concentration (Butts *et al.* 1991). Nevertheless, their removal in the low molecular weight fraction may lead to some underestimation of the actual endogenous loss of amino acids.

For the above reasons, ileal N and amino acid digestibilities were used in this thesis in studies involving effects of cottonseed CT upon protein digestion by monogastric animals. Apparent ileal digestibility was determined in all monogastric experiments, whilst true ileal digestibility and endogenous protein loss were determined in some experiments. All monogastric experiments used the laboratory rat and in the final experiment the rat was compared with the pig for studying the effects of cottonseed CT upon protein digestion.

1. 10. 2 Measurement of Protein Solubilization and Degradation in the Rumen

The value of a protein fed to ruminants is influenced substantially by the extent to which it is digested in the rumen. The digestion of dietary protein in the rumen can be attributed to the combined processes of solubilization and degradation. Solubilization is the release of protein from plant cells into the fluid phase of the rumen following chewing and is an important prerequisite for degradation (Hungate 1966), whilst degradation is the catabolism of protein by microbial proteases resulting in the release of amino acid and NH_3. Between 20 and 100% of the protein in many diets based on high protein forages, protein meals and grains may be soluble. Protein concentrate meals such as CSM are of most value in ruminant nutrition if the rumen degradability is low (ie undegradable protein as a proportion of crude protein is high).

It has been proposed that the solubility of protein-N in buffer solution can be used as an index of degradability of protein meals in the rumen (Preston and Leng 1987). However, soluble proteins such as serum albumin, ovalbumin, chloroplast protein extract and soluble proteins from soya bean meal and rapeseed meal have variable resistance to degradation in the rumen (Mahadevan *et al.* 1980). There are several techniques (both laboratory and animal) that have been used to estimate rumen protein solubility and degradability, which include the *in vitro* total N solubility, *in vitro*

enzymatic techniques, *in vitro* rumen inoculum, *in situ* techniques and the *in vitro* incubation with rumen fluid, followed by fractionation of individual proteins using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and their quantification using imaging densitometry.

Many solvents varying in complexity have been employed for the purpose of determining *in vitro* soluble N. Solubility of total N *in vitro* is dependent on the chemical characteristics of the buffer, ionic strength, temperature, and pH (Waldo and Goering 1979). Differences between laboratories were due to differences in extraction time, degree of agitation, feed particle size and solvent to feed ratio. *In vitro* N solubility is highly correlated to short-term (1 to 3 h) rumen digestion of protein (r=0.80-0.97; Crawford *et al.* 1978; Madsen and Hvelplund 1985), not to extent of rumen digestion (Nocek 1988).

Digestion systems utilising proteolytic enzymes offer several advantages over live microbial cultures for measuring *in vitro* rumen protein digestion (low cost, time reduction, less contamination of feed residue, no cannulated animal required). However, enzymatic specificity of commercial enzyme preparations in relation to rumen proteolytic activity may be different and becomes an important factor. Several studies have employed single enzyme systems of non-bacterial origin including pepsin, trypsin, papain and pronase (Pion *et al.* 1983). Other studies have utilised broad specificity fungal and bacterial enzyme sources (Poos-Floyd *et al.* 1985) or cell coat protease from mixed rumen isolates (Russell *et al.* 1981) to estimate rumen protein degradability. Several studies have found good correlation (r=0.61-0.94) between rumen protein degradability using single enzyme incubation techniques and *in vivo* techniques (Pion *et al.* 1983; Poncet *et al.* 1983; Krishnamoorthy *et al.* 1983). In general, proteolytic enzyme systems have potential for estimating rumen protein digestion, and may be more suitable for measuring relative differences between feedstuffs than providing absolute values. However, potential sources of variation include buffer pH and composition, duration of incubation, enzyme saturation conditions and incubation temperature.

Several researchers (Little *et al.* 1963; Broderick 1978, 1982; Broderick *et al.* 1980; Raab *et al.* 1983) used ammonia release to predict protein digestion *in vitro*. Broderick (1982) showed negative ammonia release values for sorghum grain and corn after 2 h of incubation. Negative ammonia release occurs when feed contains a considerable amount of fermentable carbohydrate. Protein degradation will be underestimated because of the ammonia used for microbial growth (Broderick 1978; Chamberlain and Thomas 1979). Broderick (1978) refined the procedure by adding

hydrazine sulfate to inhibit uptake of amino acids and ammonia by bacteria. This procedure requires analysis of the incubation material for both ammonia and total amino acids to calculate degradation. Although this procedure has been applied to typical feedstuffs (Broderick 1984), interpretation of results may limit its usefulness with many feeds. Raab *et al.* (1983) and Menke *et al.* (1979) developed an approach whereby protein degradation was based on production of ammonia and gas when increments of starch were provided for microbial synthesis. The procedure integrates the relationship between fermentation of carbohydrates and microbial protein synthesis for determination of NH_3N incorporated into microbial protein. A potential concern is the type of carbohydrate source (fermentability) used in relation to protein source (degree of protection) with regard to gas production (Nocek 1988).

The suspension of feed materials in the rumen (e.g. *in situ* technique, *in sacco* technique, artificial fibre bag technique) allows intimate contact of the test feed with the rumen environment. There is no better way to simulate the rumen environment within a given feeding regimen (temperature, pH, buffer substrate, enzymes), although in the rumen environment, the feed is not subjected to the total rumen experience: i.e., mastication, rumination and passage. This technique has been used since late 1970s or early 1980s (Mehrez and Ørskov 1977; Ørskov and McDonald 1979) and is the basis for predicting rumen protein digestion in several feeding systems (Chalupa 1975; NRC 1985; Waldo and Glenn 1984). The *in situ* protein digestion in the rumen is estimated using the following equation (Ørskov and McDonald 1979)

$$Y = A+B\ (1-e^{-ct}) \qquad (1)$$

where Y represents percent protein digestion at time (t) in hours spent in the rumen. The constants A, B and C represent, respectively, the instantly soluble fraction (A), the proportion digested in time t (B) and the digestion rate of the 'B' fraction (C). Potential digestibility is calculated as $A+B$. Predicted digestibility (P) is estimated from the equation of Ørskov and McDonald (1979)

$$P = A + [BC/(C+k)] \qquad (2)$$

where k is the rumen particulate fractional outflow rate. Predicted digestibility thus gives an estimate of rumen digestibility at a specified rumen outflow rate.

However, its increased popularity has also subjected the *in situ* technique to extensive evaluation and criticism with regard to the many inherent factors that influence digestion (i.e., bag porosity, sample size, feed particle size). In addition, bacteria can enter artificial fibre bags suspended in the rumen and contaminate the undigested

residue with microbial protein. Unless undigested residues are corrected for bacterial contamination, rumen protein digestion will be under-estimated (Chalupa and Sniffen 1994).

The loss of total N from synthetic-fibre bags using the *in situ* technique has gained wide acceptance as an index of protein degradability in the rumen. However, Spencer *et al.* (1988) reported that individual proteins (albumins) of pea seed were relatively resistant to rumen degradation, despite the almost complete loss (i.e. solubilization) of total pea seed N from synthetic-fibre bags suspended in the rumen. Therefore, rates of dietary protein solubilization and degradation in the rumen may not be similar and the loss or solubilization of total N from synthetic-fibre bags suspended in the rumen may not always be a good index of dietary protein degradation in the rumen. Protein degradation in the rumen, either *in vitro* or *in vivo* has been successfully studied by identification of individual proteins using SDS-PAGE (Nugent and Mangan 1981; Spencer *et al.* 1988; Romagnolo *et al.* 1990; McNabb *et al.* 1994) and measuring their rates of breakdown. Therefore, in this thesis protein solubility is defined as the rate of disappearance of total N from samples suspended in the rumen of sheep using the polyester bag technique (Mehrez and Ørskov 1977). Protein degradability is defined as the rate of disappearance of individual proteins during *in vitro* incubation with rumen fluid, with identification of individual proteins using SDS-PAGE.

Cottonseed contains three major types of proteins in approximately equal amounts, having sedimentation values of 2S, 5S and 9S. Protein bodies isolated from cottonseed also contain these three major proteins in similar proportions. The 5S and 9S proteins are typical globulin storage proteins, whilst the 2S proteins are albumins and are also storage protein as judged by their amino acid composition, developmental properties and high amount in the seed (Youle and Huang 1979). The 2S proteins are distinct from the 5S and 9S proteins in solubility, amino acid composition, sedimentation values, and SDS gel electrophoretic patterns. Storage proteins comprise about 70% of the total protein in cottonseed kernels, and are located within protein bodies (aleurone grains; Lui and Altschul 1967). The two principal globulin storage proteins (α-globulin and β-globulin) have been identified in cottonseed kernel, and have molecular weights of 52,000 and 48,000 Da. They have similar solubilities, ultraviolet absorption spectra, and contain similar proportions of amino acid residues (Dure and Chlan 1981). They were therefore used in this thesis in measurements of the effects of cottonseed CT upon rumen solubility and degradability of cottonseed kernel proteins.

For the reasons given above, the solubility of total N in mineral buffer solution (pH 7.0) *in vitro*, and the rate of disappearance of total N and the two major cottonseed storage proteins (52,000 and 48,000 Da) from polyester bags suspended *in situ* in the rumen of sheep were used to measure the effects of cottonseed CT upon the solubility of cottonseed kernel proteins. Degradability of the 52,000 and 48,000 Da cottonseed kernel proteins was studied using *in vitro* incubation with rumen fluid, with fractionation of the proteins using SDS-PAGE and their quantification using imaging densitometry.

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Chapter 2

CONDENSED TANNIN AND GOSSYPOL CONCENTRATIONS IN COTTONSEED AND IN PROCESSED COTTONSEED MEAL

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2.1 ABSTRACT

Experimental varieties of cottonseed and of industrial cottonseed meal (CSM) were analysed for extractable and bound condensed tannin (CT) and free gossypol. CT was present in the hulls of all varieties, with higher concentrations recorded for high tannin and glandless selections (55 and 58 g kg^{-1} DM) than for the multiple host plant resistant and high gossypol selections (38 g kg^{-1} DM). CT was present in trace amounts in the kernels (meats) of high tannin selections, but was not detected in the kernels of all other selections. Industrial CSM contained 8-15 g kg^{-1} CT, due to contamination of meats with hull components. On average for the hulls of all varieties, approximately 22, 60 and 18% of total CT was present in the extractable, protein-bound and fibre-bound forms, respectively. Total CT content in the hulls was positively correlated with the lignin content of kernels ($r=0.67$, $P<0.01$). Free gossypol was mainly found in the kernels, with negligible amounts being found in the hulls of the experimental varieties. Kernels of high gossypol selections contained higher concentrations of free gossypol (18 g kg^{-1} DM) than kernels of multiple host plant resistant, high tannin and commercial selections (10-12 g kg^{-1} DM), with free gossypol concentration being very low (0.8 g kg^{-1} DM) in the kernels of glandless cottonseed and in Australian industrial CSM. A negative correlation ($r=-0.50$, $P<0.05$) between free gossypol in the kernels and total CT in the hulls was found. The kernels of multiple host plant resistant selections were lower in neutral detergent fibre, acid detergent fibre and lignin, whilst those of the glandless selection were higher in oil, than the mean for all other selections. The results are discussed in relation to plant defence mechanisms against insect attack and in relation to the nutritive value of CSM for ruminant and monogastric livestock.

2. 2 INTRODUCTION

Approximately 90% of the world's production of oilseeds is supplied by five materials - soyabean, cotton, groundnut, sunflower and rape, with cottonseed being second in total production after soyabean (FAO 1991). Although cottonseed meal (CSM) is commonly used as a protein supplement for ruminants, its use for monogastric animals has been limited by its content of anti-nutritional factors (ANFs), such as gossypol (a plant secondary compound) and its high fibre content (Frank 1987; Lusas and Jividen 1987).

Free gossypol can be reduced to minimum levels (0.2 g kg^{-1} DM) by steaming and/or heating of cottonseed during the oil extraction process (Berardi and Goldblatt 1980; Hron *et al.* 1982), which binds gossypol to protein. High temperatures and pressures favour the formation of stable bonds between gossypol and protein, but these may be responsible for the reduced true amino acid digestibility and amino acid utilisation in CSM, especially for the amino acid lysine (Batterham *et al.* 1984; 1990; Beech *et al.* 1991).

Recent investigations by Balogun *et al.* (1990) and Terrill *et al.* (1992) have detected condensed tannins (CT), as a further group of plant secondary compounds present in commercially produced CSM. CT are recognized as being ANFs in the diets of monogastric animals, and may further contribute to the reduced protein nutritional value of CSM for monogastric animals (Huisman *et al.* 1990; Longstaff and McNab 1991). Low concentrations of CT (10-30 g kg^{-1} DM) are likely to promote increased protein "by-pass" from rumen fermentation in ruminant diets, but higher concentrations (50-100 g kg^{-1} DM) reduce feed intake and rumen fibre digestion (Barry 1989; Yu 1991).

Other potential toxic or ANFs in cottonseed products which have been reported in the literature, such as aflatoxin, phytic acid, cyclopropenoid fatty acids (CPFAs), and allergens, are outside the scope of this study, but have been discussed by Frank (1987) and Lusas and Jividen (1987).

The objectives of this study were to extend the method of Terrill *et al.* (1992), which measures extractable and bound CT, to experimental cottonseeds and industrial CSM, and also to determine the concentration of free gossypol.

2. 3 MATERIALS AND METHODS

2. 3. 1 Plant Materials

Whole seeds of 16 varieties of delinted cotton seed (100 g of each) were supplied by CSIRO Cotton Research Unit, Narrabri, Australia (Table 2. 1). These varieties were

from a series of selection programmes and are being evaluated for their cotton producing potential in Australia. Okra leaf (long narrow leaves) and glabrousness (no hair on leaves) are selection criteria to make the cotton plant less attractive to insects, and hence egg laying and subsequent larval damage are reduced (Fitt *et al.* 1992). The cotton seeds were manually separated into kernels (meats) and hulls.

Representative samples of industrial CSM and of industrial cottonseed kernels (meats) were also analysed. The industrial CSM samples were from the Narrabri and Brisbane mills of Cargill Oilseeds Ltd, Australia (pre-press solvent extraction method, using hexane as the solvent to remove oil). One random sample of meal was taken daily from each plant over a 4-day period, to provide 4 representative samples for analysis for each plant. Before chemical analysis, all samples were stored at -20°C, and then freeze-dried for 20 hours and ground to pass through a 1 mm diameter sieve.

Table 2. 1 Experimental varieties of cottonseed and their sources of origin

Samples	Plant selection criteria	Origin	Reference
MHR 10	Multiple host plant resistance	Mississippi	Jenkins *et al.* (1988a)
MHR 11	Multiple host plant resistance	Mississippi	Jenkins *et al.* (1988a)
MHR 17	Multiple host plant resistance	Mississippi	Jenkins *et al.* (1988b)
HG 063	High gossypol	Louisiana	Jones *et al.* (1988)
HG 065	High gossypol	Louisiana	Jones *et al.* (1988)
HG 660	High gossypol	Louisiana	Jones *et al.* (1988)
HT-35-5-1 Smooth	High tannin/glabrous leaf[1]	Texas	Fitt *et al.* (1992)
HT-35-5-1 Hirsute	High tannin/hairy leaf	Texas	Fitt *et al.* (1992)
HT-35-14-3	High Tannin/hairy leaf	Texas	Fitt *et al.* (1992)
CS 3810	Suspected high tannin/mite resistance	Texas	TAES (1990)
DP 90	Commercial/normal leaf	Mississippi	Fitt *et al.* (1992)
Sicala 3-3	Commercial/normal leaf	Australia	Thomson *et al.* (1990)
Siokra 1-4	Commercial/okra leaf[2]	Australia	Reid *et al.* (1989)
N74-720-199B	Okra leaf[2]/glabrous leaf[1]	Australia	Reid *et al.* (1989)
OGF Line 8	Okra leaf[2]/glabrous[1]/frego bract[3]	Australia	Thomson *et al.* (1987)
DP 16 Glandless	Normal leaf/lacks gossypol glands	USA	Fitt *et al.* (1992)

[1] Glabrous leaf; selected for no hair on leaves.

[2] Okra leaf; selected for more narrow leaf than normal cotton.

[3] Frego bract; selected for open bud, to allow better penetration of insecticides.

2. 3. 2 Condensed Tannin Analysis

The CT in cotton seeds and in industrial CSM was determined using the method of Terrill *et al.* (1992). "Free " CT in samples was extracted using a mixture of acetone:water:diethyl ether (4.7:2.0:3.3 v/v), followed by extraction of protein-bound CT using boiling sodium dodecyl sulphate (SDS; 1% w/v) containing 2-mercaptoethanol (5% w/v) in 10 mM Tris/Chloride, adjusted to pH 8.0 with HCl, (SDS solution). The CT concentration in each fraction was then determined by a modified butanol-HCl procedure. Fibre-bound CT was determined after boiling the residue remaining from the protein extraction with butanol-HCl and SDS solution. Separate standard curves using purified CT extracted from cotton leaves supplied by CSIRO, Division of Plant Industry, Canberra, were prepared in water and SDS solution, respectively and used for the analysis of extractable CT (water standards) and protein-bound and fibre-bound CT (SDS standards).

Purified CT for use as a standard was extracted from freeze dried leaves of high tannin cotton with 7:3 (v/v) acetone:water, with the acetone subsequently removed by rotary evaporation. The resultant aqueous solution was washed 3 times with diethyl ether and centrifuged (27,000 *g* for 15 min). The CT aqueous solution was freeze-dried, redissolved in 1:1 methanol:water (v/v) and purified on Sephadex LH-20. Purified CT was freeze-dried and stored in a desiccator at -20 °C until used.

The repeatability of measuring CT concentration was determined, using repeat (6 times) duplicate analysis of one sample of hulls and one sample of industrial CSM. The standard deviations (SD) and coefficients of variation (CV) for between sample and between duplicate variation were calculated. Recovery rates were determined by adding 10 mg of purified cotton CT to the extractable CT phase during the analysis of a sample of cottonseed hulls, on two occasions.

2. 3. 3 Other Chemical Analyses

Free gossypol was determined using method Ba 7-58 of the American Oil Chemists Society (1975). The total nitrogen (N) was determined by the Kjeldahl procedure, and crude protein calculated as total N x 6.25. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin were determined using the method of Robertson and Van Soest (1981). Dry matter was determined by drying at 100 °C for 24 hours and oil content was determined by extraction with petroleum ether (BPt 40-60 °C) for 8 hours.

2. 4 RESULTS

2. 4. 1 Condensed Tannin

Typical standard curves of purified cotton CT in water or in SDS solution are shown in Figure 2. 1. Both curves were linear up to concentrations of 700 ug CT/ml, but lower absorbances were observed in SDS solution than in water.

The repeatability of the methods (coefficients of variation) for measuring extractable and bound CT in cottonseed hulls and industrial CSM are shown in Table 2. 2. Repeatability was generally high, but both between sample and between duplicate variation increased from the determination of free CT, to protein-bound CT, to fibre-bound CT. Variation was greater in the sample of industrial CSM than in the sample of seed hulls. The mean recovery of purified CT added to the extraction step was 106% (n=2).

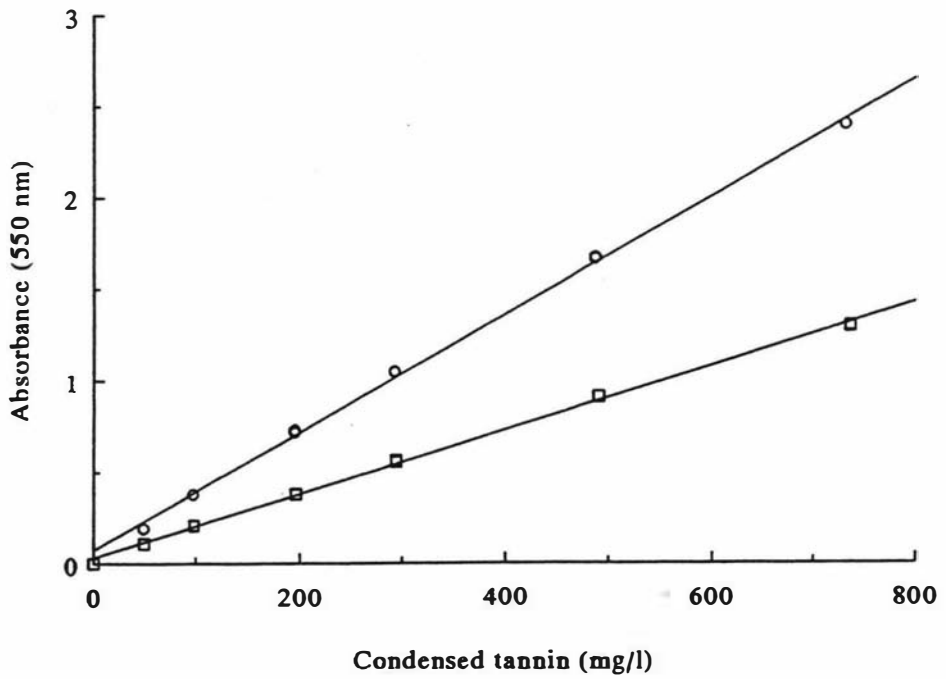

Fig 2. 1 Standard curves of absorbance (at 550 nm) vs concentration of purified condensed tannin from cotton leaves, determined using the butanol-HCl procedure of Terrill *et al.* (1992). O, condensed tannin made up in aqueous solution; □, condensed tannin made up in SDS solution containing 2-mercaptoethanol.

Table 2. 2 Repeatability of measurements for extractable and bound condensed tannins in a sample of cottonseed hulls and in a sample of industrial processed cottonseed meal

	Extra-ctable	Protein-bound	Fibre-bound	Total
Cottonseed hulls[1]:				
Mean concentration (% DM)	13.2	28.4	6.6	48.1
Between sample variation:				
SD	0.51	0.64	0.65	1.03
CV (%)	3.88	2.24	9.96	2.15
Between duplicate variation:				
SD	0.47	1.60	0.94	1.73
CV (%)	3.56	5.63	14.27	3.60
Processed cottonseed meal[1]:				
Mean concentration (% DM)	1.7	7.7	3.2	12.6
Between sample variation:				
SD	0.16	0.89	1.15	1.03
CV (%)	9.53	11.71	35.48	8.17
Between duplicate variation:				
SD	0.13	0.98	0.77	1.43
CV (%)	7.80	12.78	24.14	11.36

[1] Six independent measurements per sample.

CT was detectable in trace amounts (0.5-0.6 g kg^{-1} DM) in the kernels of the high tannin cottonseed varieties, but was not detected in the kernels of all other varieties. Total CT concentrations in the hulls of cottonseeds ranged from 32 to 65 g kg^{-1} DM (Table 2. 3), with the highest values found for the high tannin selection (50-65 g kg^{-1} DM) and the glandless selection (58 g kg^{-1} DM). The multiple host plant resistant and high gossypol selected varieties had relatively lower amounts of CT in the hulls (38 g kg^{-1} DM overall). Okra leaf varieties had the lowest concentration of fibre-bound CT, 14% of total CT on average. In these16 varieties, 22, 60 and 18% of hull CT was in the free, protein-bound and fibre-bound fractions, respectively.

Total CT in the hulls (mg $seed^{-1}$) of experimental varieties was positively correlated ($r= 0.67$; $P<0.01$) with lignin in the kernels (mg $seed^{-1}$; Figure 2. 2), but no significant correlation between the lignin in hulls and total CT of hulls was found.

Industrial CSM contained measurable concentrations of CT (Table 2. 3), with 50-62% being found in the protein-bound fraction and 37-39% found in the fibre-bound fraction. Industrial CSM from the Narrabri mill had higher CT concentrations compared with industrial CSM from the Brisbane mill. Cottonseed meat produced from the Narrabri mill contained very low concentrations of CT (Table 2. 3), with

approximately 50% of measurable CT being extractable with a mixture of acetone/water/diethyl ether.

Table 2. 3 The concentrations[1] of extractable and bound condensed tannin (g kg^{-1} DM) in the hulls of experimental cottonseed varieties and in industrial cottonseed meal

Plant selection criteria	Extra-ctable	Protein-bound	Fibre-bound	Total (calculated)
		Cottonseed		
Multiple host plant resistant:				
MHR 10	7.0	26.8	7.0	40.8
MHR 11	10.0	23.8	5.4	39.3
MHR 17	6.5	21.6	6.1	34.1
High gossypol:				
HG 063	6.2	22.2	9.5	37.9
HG 065	7.4	24.5	7.7	36.9
HG 660	8.1	21.1	8.3	37.4
High tannin:				
HT-35-5-1 Smooth	9.8	31.3	8.7	49.8
HT-35-5-1 Hirsute	12.1	30.2	10.1	52.4
HT-35-14-3	15.7	38.2	11.4	65.3
CS 3810	7.3	33.0	10.2	50.5
Commercial or Australian-bred cultivars:				
DP 90	8.4	19.5	5.3	33.3
Sicala 3-3	4.7	21.5	6.2	32.4
Siokra 1-4	14.5	28.4	6.6	49.5
N74-720-199B	9.9	22.5	4.6	37.0
OGF Line 8	13.4	28.7	7.1	49.2
Glandless:				
DP 16	15.3	32.5	9.9	57.7
		Industrial cottonseed products		
Commercial CSM A[2]	2.1	7.6	5.4	15.1
Commercial CSM B[3]	0.1	4.9	2.9	7.9
Commercial cottonseed meat[2]	1.1	0.7	0.5	0.23

[1] Mean of duplicate determinations.

[2] Samples were from Narrabri cottonseed processing plant, NSW, Australia.

[3] Samples were from Brisbane cottonseed processing plant, QLD, Australia.

2. 4. 2 Free Gossypol

The repeatability of the method for measuring free gossypol was high. The standard deviation (SD) and coefficient of variation (CV) for between duplicate variation in samples of CSM were 0.002 and 3.2% respectively (n=8).

Free gossypol in the kernels of the experimental cottonseeds and industrial cottonseed products is shown in Table 2. 4. High gossypol varieties contained the highest free gossypol (18 g kg^{-1} DM on average) in the kernels. High tannin and multiple host plant resistant varieties contained on average 12 g kg^{-1} free gossypol in the kernels, which was 35% lower than that detected in high-gossypol varieties, but 13% higher than that found in commercial or Australian-bred varieties (10.5 g kg^{-1} DM on average). Free gossypol in the kernels of the glandless variety (DP 16) was very low, but detectable (0.8 g kg^{-1} DM). The hulls of the experimental varieties contained negligible amounts of free gossypol (0.1-0.3 g kg^{-1} DM), which may have been due to contamination of the hulls with some of the kernels during sample preparation.

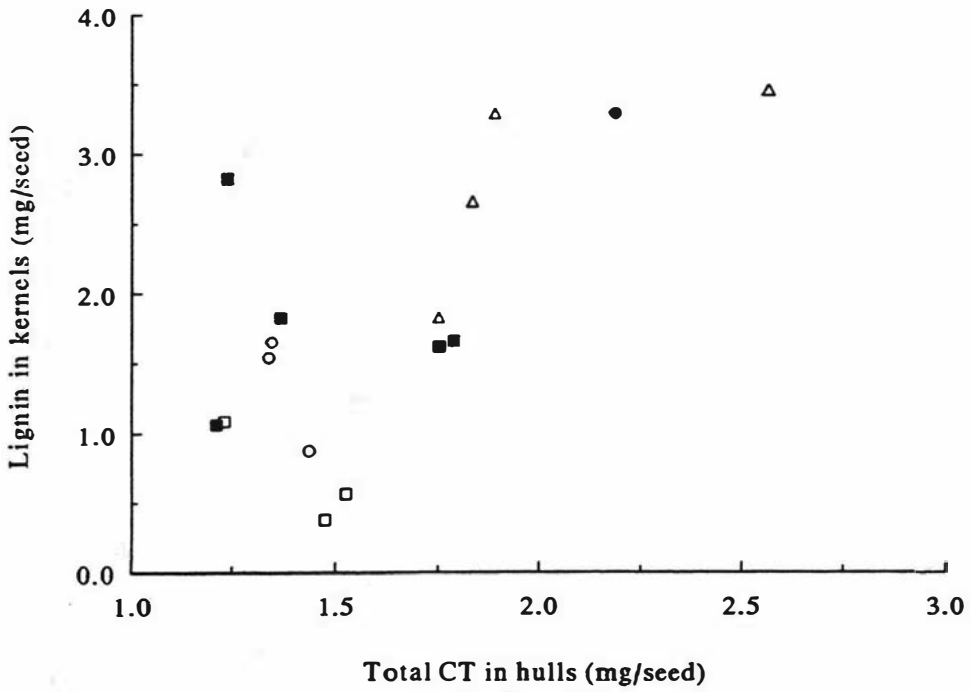

Fig 2. 2 The correlation between lignin in the kernels and total CT in the hulls of experimental cottonseed varieties. □, multiple host plant resistance; ○, high gossypol; Δ, high tannin; ■, commercial or Australian bred cultivars; ●, glandless cottonseed. r=0.67; n=16; p<0.01.

Both samples of commercial CSM contained very low concentrations of free gossypol (Table 2. 4). Commercial cottonseed meats produced at Narrabri had a free gossypol content comparable to that in the kernels of some of the experimental seed varieties.

Table 2. 4 The content[1] (g kg $^{-1}$ DM) of crude protein, oil, neutral detergent fibre (NDF), acid detergent fibre (ADF), lignin and free gossypol in the kernels of cottonseed of experimental varieties and in processed cottonseed products

Samples	Crude protein	Oil	NDF	ADF	Lignin	Free gossypol
			Cottonseed kernels (Meats)			
Multiple host plant resistant:						
MHR 10	354	366	142	55	9	11.9
MHR 11	338	375	133	36	6	10.5
MHR 17	349	336	184	73	17	13.4
High gossypol:						
HG 063	349	337	219	103	14	17.9
HG 065	341	348	242	102	26	18.9
HG 660	342	351	254	103	24	18.4
High tannin:						
HT-35-5-1 Smooth	334	344	221	104	53	13.2
HT-35-5-1 Hirsute	344	341	252	129	41	12.5
HT-35-14-3	331	344	281	129	57	10.4
CS 3810	351	342	258	90	28	10.3
Commercial or Australian-bred cultivars:						
DP 90	363	342	212	74	47	12.0
Sicala 3-3	370	342	208	73	17	10.2
Siokra 1-4	350	337	252	95	25	9.0
N74-720-199B	334	373	276	109	29	9.9
OGF Line 8	339	343	236	107	26	11.6
Glandless:						
DP 16	326	392	280	129	53	0.8
			Commercial cottonseed products			
Commercial CSM A[2]	429	36	269	183	58	0.8
Commercial CSM B[3]	468	104	237	122	54	0.7
Commercial cottonseed meat[2]	344	328	317	161	75	12.2

[1] Mean of duplicate determinations.

[2] Samples were from Narrabri cottonseed processing plant, NSW, Australia.

[3] Samples were from Brisbane cottonseed processing plant, QLD, Australia.

Free gossypol in the kernels of experimental varieties (mg/seed) was negatively correlated ($r=-0.50$; $p<0.05$) with total CT in the hulls (mg/seed) of experimental varieties (Figure 2. 3). Selection for high or low gossypol in cottonseed kernels tended to produce varieties with respectively low and high levels of CT in the hulls.

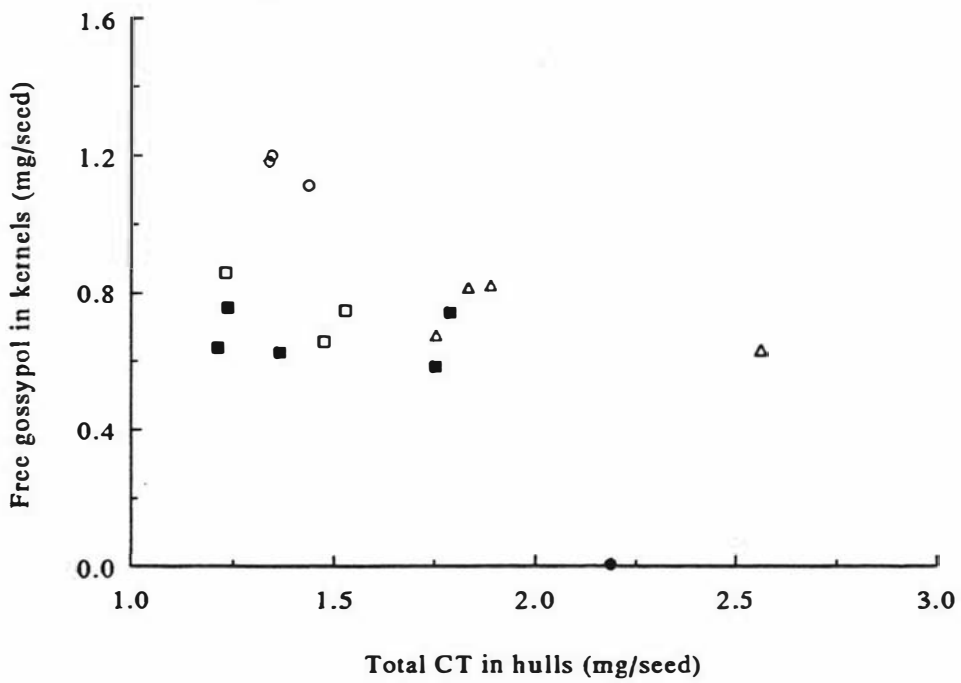

Fig 2. 3 The correlation between free gossypol in the kernels and total CT in the hulls of experimental cottonseed varieties. □, multiple host plant resistance; ○, high gossypol; Δ, high tannin; ■, commercial or Australian bred cultivars; ●, glandless cottonseed. $r= -0.50$; $n=16$; $p<0.05$.

2. 4. 3 Crude Protein, Oil and Fibre

The crude protein (CP), oil, neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin contents of the kernels of cottonseed are shown in Table 2. 4. The CP and oil contents of the kernel of the DP 16 glandless were 2% lower and 5% higher, respectively, than the mean levels (346 g kg^{-1} and 348 g kg^{-1}) for the rest of the varieties. Kernels of the three multiple host plant resistant varieties had notably lower

contents of NDF, ADF and lignin than kernels of the other varieties. Also, kernels from the high tannin varieties tended to have higher lignin levels than kernels from the other varieties.

There were very few differences in the CP, oil, NDF, ADF and lignin contents of the hulls from the experimental cottonseeds (Table 2. 5). In all varieties hull vs seed weight was similar (Table 2. 5).

Table 2. 5 The content[1] (g kg^{-1} DM) of crude protein, oil, neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin in the hulls of cottonseed of experimental varieties

Plant selection criteria	% of seed weight	Crude protein	Oil	NDF	ADF	Lignin
Multiple host plant resistant:						
MHR 10	374	42	16	884	608	230
MHR 11	376	42	15	877	606	254
MHR 17	361	38	14	903	624	236
High gossypol:						
HG 063	379	36	13	899	618	232
HG 065	365	35	9	906	636	247
HG 660	358	37	11	910	634	238
High tannin:						
HT-35-5-1 Smooth	380	31	12	894	613	250
HT-35-5-1 Hirsute	350	35	12	888	618	247
HT-35-14-3	392	35	15	878	596	234
CS 3810	347	39	11	882	635	245
Commercial or Australian-bred cultivars:						
DP 90	372	35	11	901	590	216
Sicala 3-3	374	38	9	904	603	231
Siokra 1-4	354	34	9	891	618	232
N74-720-199B	370	38	15	885	613	208
OGF Line 8	364	36	12	882	604	229
Glandless:						
DP 16	379	38	12	872	625	238

[1] Mean of duplicate determinations.

Industrial CSM from Narrabri had a greater fibre content than that from Brisbane (Table 2. 4). NDF, ADF and lignin of Narrabri CSM were 14%, 50% and 7% higher than those of Brisbane CSM. Industrial cottonseed meat contained higher fibre compared with the kernels from experimental varieties. The oil content, however, of

industrial cottonseed meat was 2% lower than the average level of the experimental cottonseed varieties.

2. 5 DISCUSSION

The present results demonstrate that the method of Terrill *et al.* (1992) for measuring extractable and bound CT in forages can be extended to cottonseeds and cottonseed products. A complete recovery of added purified CT (106%) was found. Lower absorbances for purified cotton CT made up in SDS solution compared with water were found in the present study, similar to the results of Terrill *et al.* (1992) for CT extracted from the forages *Lotus pedunculatus* and *Hedysarum coronarium.* This can be corrected for by using the aqueous standard curve for measuring "free" CT and the SDS standard curve for measuring protein-bound and fibre-bound CT. The low amounts of cotton CT detected in the initial acetone:water:diethyl ether extract and the high amounts of CT detected as protein-bound and fibre-bound CT show that a bound CT method is essential to reliably measure CT in cottonseed and cottonseed products.

CT in cottonseed was estimated using CT extracted from cotton leaf as a standard, and it is possible that degree of polymerization may differ in CT from the two sources. However, as the butanol/HCl reaction involves hydrolysis of CT to monomer units and formation of anthocyanidin from a portion of these, it is unlikely that differences in chain length would significantly influence the results, as found by Porter *et al.* (1986).

Repeatability of measurements between samplings and between duplicates was acceptable. Whilst samples of the 16 experimental cottonseeds were not replicated, the precision of the method suggests that differences found between selection lines (varieties) are probably real. Greater variation in CT detected in the protein-bound and especially the fibre-bound steps with industrial CSM is probably due to CT only being present in hulls, and represents sampling variation from a mixture of meats and hulls.

The cottonseed kernels contained negligible amounts of CT, with almost all of the CT being found in the hulls. Kernels from the high tannin varieties tended to be high in lignin (also defined as a secondary compound), which can be explained by lignin and CT being produced by related biochemical pathways (Figure 2. 2; Barry 1989). However, no significant correlation between total CT and lignin contents in the cottonseed hulls was found in this study. Gossypol, CT and lignin probably evolved in cotton as chemical defences against insect attack. From the results of the present study it seems that selection for high levels of CT or gossypol has led to corresponding reductions in the other, with high and low (ie glandless) gossypol selection producing seed hulls low and high in CT, respectively (Figure 2. 3).

Fitt *et al.* (1992) showed reduced larval growth on the leaves of both high tannin and high gossypol selections, with the latter showing most promise, and recommended this factor be incorporated into selection programmes for Australian cotton. Selection for high tannin to control insects poses no animal nutrition problems because CT only occurs in the hull and manufacturing processes can be devised to remove most of this component. However, as gossypol occurs in the kernels, as well as in leaves and other plant parts, selection for high gossypol in insect control programmes will probably cause additional nutritional problems, especially for monogastric species.

Recent studies have shown that erythrocyte fragility in feeder lambs (Calhoun *et al.* 1990) and heifers (Gray *et al.* 1990) was increased by feeding moderate amounts of cottonseed products containing free gossypol. Smalley and Bicknell (1982) reported case studies of gossypol toxicosis in mature dairy cattle fed relatively high amounts of ammoniated whole cottonseed. These findings in ruminants indicate that ingestion of free gossypol at high levels may overwhelm ruminal detoxification and hence result in the absorption of quantities of free gossypol that may be potentially toxic. More than 95% of total gossypol in dehulled cottonseed kernels was free gossypol (Pons and Eaves 1967; Ikurior and Fetuga 1984). The free gossypol contained in the cottonseed kernels becomes bound to other seed components and/or is destroyed during seed processing (Ikurior and Fetuga 1984).

There was little difference among the protein contents of the kernels, whereas the kernel of glandless cottonseed contained a higher concentration of oil than the kernels from the other varieties, which was similar to the findings of Lawhon *et al.* (1977). Kernels of the three multiple host plant resistant varieties had notably lower contents of total fibre and lignin than kernels made from the other varieties, and this should result in improved digestion in monogastric animals. However, these selections have shown very little resistance to insect egg laying and larval damage under Australian conditions (Fitt *et al.* 1992).

Industrial CSM produced at both the Narrabri and Brisbane plants contained CT, probably due to contamination of the kernels with parts of the hulls under large scale manufacturing. The higher concentrations of CT in CSM from the Narrabri mill is probably due to the practice of leaving some hulls with the kernels, to facilitate passage through the extruders prior to solvent extraction of the remaining oil. According to the CT concentrations, CSM produced at Narrabri and Brisbane contained approximately 300 and 150 g kg^{-1} of hulls, respectively. The tannin to protein ratio in Narrabri and Brisbane CSM was 0.04 and 0.02, respectively. Terrill *et al.* (1992) also found 0.04 g CT per 100 g crude protein in cottonseed meals. The small amounts of CT detected in

the industrial cottonseed meats from Narrabri indicates that approximately 5% of the original hulls were left in the meats after decortication.

CT may be a further contributing factor to the reduced true amino acid digestibility in CSM diets for monogastrics (Batterham *et al.* 1990; Beech *et al.* 1991) and to the high degree of rumen "by-pass" of CSM protein in ruminant diets (Lee *et al.* 1987). The interactions between CT, gossypol and heat need to be evaluated in future studies for their effects upon protein utilization, to define methods of manufacturing that yield optimum protein nutritive value for both ruminant and monogastric animals.

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Chapter 3

EFFECT OF BOUND CONDENSED TANNIN FROM COTTONSEED UPON *IN SITU* PROTEIN SOLUBILITY AND DRY MATTER DIGESTION IN THE RUMEN

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3. 1 ABSTRACT

The effect of adding cottonseed hulls upon the solubility of protein in unheated solvent extracted cottonseed kernels was studied using both *in vitro* incubation in mineral buffer and the *in situ* polyester bag technique. The latter technique was also used to study effects on rumen dry matter (DM) digestion. Effects attributable to condensed tannin (CT) were assessed by making measurements in the presence and absence of polyethylene glycol (PEG; MW 3,500), which binds and inactivates CT. Cottonseed hulls contained 51 g CT kg^{-1} DM, with 56 and 20% of the total CT being bound to protein and fibre, respectively; no CT was detected in kernel. Hulls and extracted kernel contained 33 and 509 g protein kg^{-1} DM, and 887 and 289 g fibre kg^{-1} DM. In the absence of hulls, 42% of the total nitrogen (N) in cottonseed kernel was soluble in mineral buffer *in vitro*, whilst potential *in situ* N solubility and predicted rumen N solubility (corrected for rumen outflow rate) were 99 and 86% respectively. Addition of hulls linearly reduced both *in vitro* N solubility and potential *in situ* N solubility, with 100% hulls addition reducing potential N solubility and predicted rumen N solubility to 94 and 79% respectively. PEG addition had no effect upon the protein solubility of kernels, but increased N solubility in mixtures of hulls and kernels *in vitro* but not *in situ*. Two mg PEG mg^{-1} total CT was shown to reverse the effect of CT in reducing *in vitro* protein solubility. Potential *in situ* DM digestion and predicted rumen DM digestion (corrected for rumen outflow) were substantially lower for cottonseed hulls (41 and 33%) than for kernels (99 and 88%). Increasing the addition of hulls to kernels lowered the rumen DM digestion of mixtures in a quadratic manner, with increasing rate of hulls causing progressively smaller depressions. Addition of PEG had no effect upon the digestion of kernel DM, but increased potential DM digestibility and predicted rumen DM digestion of hulls to 47 and 40% respectively, and also produced an increase in mixtures of hulls and kernels. It was concluded that the high protein solubility of unheated solvent extracted cottonseed kernels can be linearly reduced by the addition of cottonseed hulls, with the magnitude of the reduction being small, and that the presence of bound CT in hulls substantially depressed fibre digestion by rumen micro-organisms. It is doubt full that CT plays a significant role in the reduction of rumen protein solubility produced by cottonseed hulls.

3. 2 INTRODUCTION

Solvent extracted cottonseed meal (CSM) has long been recognized as a protein supplement for ruminant and monogastric livestock. Free gossypol, a polyphenolic binaphthyl aldehyde contained in the seed pigment glands, is considered one of the major anti-nutritional factors (ANFs) in CSM. In CSM the concentration of free gossypol is influenced by the method of oil extraction, with high temperatures and pressures causing "binding" of gossypol with the ε-amino group of lysine, thus reducing free gossypol concentration (<0.2 g kg^{-1} DM; Berardi and Goldblatt 1980). Toxicity symptoms attributed to gossypol have been reported primarily in non-ruminants. Ruminants appear to be less susceptible to gossypol toxicity; apparently due to their ability to detoxify gossypol by binding with soluble proteins present in the rumen fluid (Reiser and Fu 1962), except for high intake of free gossypol, which can overwhelm this protective system, leading to toxicoses in adult ruminants (Lindsey *et al.* 1980; Risco *et al.* 1993).

Recent investigations have found another group of plant secondary compounds, condensed tannins (CT), present in commercially produced CSM (Balogun *et al.* 1990; Terrill *et al.* 1992; Yu *et al.* 1993). These have been shown to originate from cottonseed hulls, where they occur mainly bound to protein and fibre, whilst cottonseed kernel does not contain CT (Yu *et al.* 1993). CT are recognized as being an ANF in monogastric animal diets (Huisman *et al.* 1990), but in ruminants low concentrations may improve the efficiency of protein digestion by forming hydrogen bonded complexes with proteins (Barry 1989). The protein-CT complexes are stable and insoluble at rumen pH (5.5-7.0) but dissociate and release protein below pH 3.0 (Jones and Mangan 1977). Waghorn *et al.* (1987) showed that the action of CT in the forage of *Lotus corniculatus* increased the absorption of essential amino acids from the small intestine of sheep by 62%. Barry (1989) concluded that low concentrations (10-30 g kg^{-1} DM) of CT in forage diets are likely to increase amino acid absorption from the small intestine, but that higher concentrations reduced both feed intake and rumen fibre digestion.

There is less information available on the effects of CT on rumen solubility/degradability of protein concentrate meals, particularly for oil-seed meals. Objectives of the present study were to determine the effect of CT in cottonseed hulls upon the *in vitro* and *in situ* solubility of total nitrogen (N) and two individual proteins (52,000 and 48,000 Da molecular weight) in unheated solvent extracted cottonseed kernel, and upon the *in situ* dry matter (DM) digestion of cottonseed hulls and mixtures of kernels and hulls. The 52 and 48 kDa proteins are two principal storage proteins,

and the storage proteins comprise about 70% of the total protein in cottonseed kernel (Dure and Chlan 1981).

The loss of DM and total N from polyester bags immersed in rumen fluid *in vitro* and *in situ* has had wide acceptance as an index of DM and N degradability in the rumen (Mehrez and Ørskov 1977; Crooker *et al.* 1978; Kempton 1981). However, Stern and Satter (1984) compared protein solubility in a mineral buffer solution with *in situ* rumen protein degradation, measured as disappearance from dacron-fibre bags, and obtained a correlation coefficient of only 0.26. Spencer *et al.* (1988) reported that individual pea seed proteins were relatively resistant to rumen degradation, despite the almost complete loss of total pea seed-N from synthetic-fibre bags suspended in rumen fluid *in vitro*. Protein solubilization is an important prerequisite for rumen degradation (Hungate 1966), however the rates of solubilization and degradation of protein in the rumen are not always similar (McNabb 1990). Therefore, in this paper, protein solubility is defined as the extractability of total N in mineral buffer solution (pH 7.0) *in vitro*, and also as the rate of disappearance of total N and individual storage proteins from samples suspended in the rumen of sheep using the polyester bag technique (Mehrez and Ørskov 1977). Protein degradability is defined as the rate of disappearance of individual storage proteins during *in vitro* incubation with rumen fluid, with identification of individual proteins using sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The effect of hulls and CT upon the degradability of cottonseed kernel proteins is described by Yu *et al.* (1995a). Loss of DM from polyester-fibre bags is defined as *in situ* rumen DM digestion.

3. 3 MATERIALS AND METHODS

3. 3. 1 Experimental Design

Three experiments were conducted to determine the effect of CT in cottonseed hulls upon the *in vitro* and *in situ* protein solubility and *in situ* DM digestion in cottonseed kernels. All investigations were done with seeds of *Gossypium hirsutum*, var. Siokra L22, as supplied by Cotton Seed Distributors Ltd, Wee Waa, NSW, Australia. The effects of CT, in the present study, were assessed by making measurements in the presence and absence of polyethylene glycol (PEG, molecular weight (MW) 3,500, Union Carbide, Danbury, CT, USA). PEG binds strongly to CT and can be used to displace protein from the CT-protein complexes (Jones and Mangan 1977; Barry and Manley 1986). Therefore, effects of CT can be quantified by comparing controls (CT acting) with PEG treatments (CT inactivated).

In Experiment 1 the effect of CT in cottonseed hulls upon the *in vitro* solubility of total N in cottonseed kernel was determined. Experiment 2 investigated the *in situ* solubility of total N and of two individual storage proteins in cottonseed kernels; and the *in situ* rumen DM digestion in cottonseed kernel and mixtures of kernel and hulls. Experiment 3 determined the effect of bound CT upon the *in situ* rumen DM digestion of cottonseed hulls.

3. 3. 2 Preparation of Cottonseed Kernel and Hulls

Delinted whole cottonseed was cracked by passage through a Crushing-Mill (AB Thorell & Persson, Uppsala, Sweden), and then separated into kernels and hulls using an air-blow technique, at the Seed Technology Centre, Massey University.

Separated kernels were freeze-dried for 2 days, and then ground to pass through a 2 mm diameter sieve. Ground kernels were extracted in 1 kg batches by continuous stirring with pure hexane (1:2 w/v) for 6 h at ambient temperature, according to the method of Pons and Eaves (1967). After displacement washing with hexane (5 × 1,000 ml), using vacuum filtration, the extracted meal was air-dried, and ground to pass through a 1 mm sieve. Gossypol was then extracted, with minimal removal of protein using the following conditions: acetone in water (70:30, w/w, as the solvent); temperature 30°C; extracting time 30 min; solvent to kernel ratio 2:1; displacement washing two times. The resulting meal from the second extraction was air-dried again at ambient temperature.

Separated hulls were ground to pass through a 1 mm diameter sieve. Fully extracted cottonseed kernel and ground hulls were stored at -20°C until used. The chemical composition of cottonseed kernel before and after extraction and of cottonseed hulls is shown in Table 3. 1. Total CT in ground hulls comprised 24% extractable CT, 56% protein-bound CT and 20% fibre-bound CT.

3. 3. 3 Experiment 1

In Experiment 1a, the effect of different concentrations of PEG (1, 2, 3, 4 and 5 mg mg^{-1} total CT) upon total N solubility in a mixture of 1 g solvent extracted cottonseed kernel plus 200% cottonseed hulls (ie 2 g) was determined. The mixture contained: 194 g kg^{-1} DM of crude protein; 34 and 8.3 g kg^{-1} DM of total and extractable CT, respectively. Experiment 1b investigated the effect of CT in cottonseed hulls upon *in vitro* total N solubility of cottonseed kernel. The treatments comprised 1 g of solvent extracted cottonseed kernel plus 0, 25, 50, 100 and 200% of ground cottonseed hulls,

with or without 2 mg PEG mg^{-1} total CT. The crude protein and CT contents in each treatment are shown in Table 3. 1.

Table 3. 1 The chemical composition[1] (g kg^{-1} DM) of cottonseed kernel, hulls and mixtures used in the experiments

	Dry matter	Crude protein	Oil	NDF	ADF	Lignin	Free gossypol	Total CT
Cottonseed kernel:								
untreated	938	360	387	198	63	37	12.6	0
extracted[b]	909	509	138	289	137	52	0.95	0
Cottonseed hulls	900	33	9	887	734	153	0.23	51.0
Extracted kernel[b]/hull mixtures:								
+25% hulls	914	420	-	-	-	-	0.87	10.2
+50% hulls	907	354	-	-	-	-	0.76	17.0
+100% hulls	908	269	-	-	-	-	0.58	25.5
+150% hulls	905	223	-	-	-	-	0.47	30.6
+200% hulls	904	194	-	-	-	-	0.42	34.0

[1] Mean of duplicate determinations.

[2] Hexane+acetone

Phosphate buffer (pH 7.0) was prepared by mixing 195 ml of 0.15 M NaH_2PO_4 with 305 ml 0.15 M Na_2HPO_4 and making up to 1 litre with distilled water. Freshly prepared phosphate buffer (50 ml, pH 7.0), maintained at 39°C, was added to groups of volumetric flasks (250 ml); 1 g of unheated solvent extracted cottonseed kernel, and the required amounts of ground cottonseed hulls and PEG were then added. Flasks were fitted with Bunsen valves and incubated in a shaking water bath (90 rpm) at 39°C for 2 hours. The mixture was centrifuged at 27,000 × *g* for 15 min and total N content determined on 10 ml of the clear supernatant solution. All treatments were done in triplicate.

3. 3. 4 Experiments 2 and 3

3. 3. 4. 1 Animals and Diet

In Experiments 2 and 3, two groups each of six adult male castrated sheep (mean liveweight 59 kg, SE 1.9), fitted with a rumen cannula (55 mm ID) were maintained on a basal diet of 900 g d^{-1} of meadow hay (20 g kg^{-1} N, 890 g kg^{-1} DM) supplemented with 250 g d^{-1} of lucerne chaff (30 g kg^{-1} N, 860 g kg^{-1} DM), offered at hourly intervals, from overhead belt-feeders, for at least 2 weeks before the experiments commenced.

Water was provided and also a multimineral salt block (Summit®, NZ) was freely available. All sheep were drenched with an anthelmintic to control internal parasites (12 ml Ivomec; Merck Sharp and Dohme (NZ) Ltd) and were treated for lice (10 ml Wipeout; Coopers Animal Health (NZ) Ltd) before the experiments commenced.

In both Experiments, one group of six sheep (PEG sheep) received an intraruminal infusion of PEG at a rate of 25 g d^{-1} (in 240 ml water) in all experimental periods, whilst the remaining group of six sheep (Control sheep) received an intraruminal infusion of the same volume of water. There was a two day rest period between the two experiments.

3. 3. 4. 2 Experimental Procedures

In Experiment 2, the rate of *in situ* DM digestion and N solubility of test feeds was measured using the polyester bag method of Mehrez and Ørskov (1977). Test feeds incubated in the bags were unheated solvent extracted cottonseed kernel mixed with 0, 25, 50, 100, 150 and 200% of ground cottonseed hulls. The experiment was run over six consecutive periods, each of 2 days, with the six test feeds rotated among the six sheep in each group (Control sheep and PEG sheep), according to a paired 6 × 6 Latin square design. Control sheep comprised one Latin square and PEG sheep comprised a second Latin square.

Six polyester bags (Estal-mono, 47 μm pore size, Swiss Screens, Sydney, Australia) measuring 7×14 cm internally and having rounded comers, each containing a marble (approximately 5 g) to ensure that the bags would not float in the rumen, plus approximately 5 g of air dried test feed, were lowered into the rumen of each sheep. Bags were removed from the rumen after 4, 8, 12, 24, 36 and 48 h incubation and were rinsed with tap water until the rinse fluid was clear. Bags with their contents were then dried in a forced-draught oven at 60°C for 48 h, cooled in a desiccator and weighed. Loss in dry weight was reported as *in situ* DM digestibility. Dried residue of the test feeds in the bags was then analysed for total N. DM and N disappearance in the rumen due to simple solubilization was estimated by soaking control bags in water in a shaking water bath (90 rpm) at 39°C for 2 h (Kempton 1981). This represented initial solubility (t=0).

In Experiment 3, six Control and six PEG-infused sheep were used in one time period to determine the *in situ* DM digestion of cottonseed hulls. Six polyester bags each containing a marble and approximately 3 g of hulls were suspended in the rumen of each sheep. Bags were removed from the rumen after 4, 8, 12, 24, 36 and 48 h incubation. The DM disappearance of hulls at (t=0) was determined, and the residues from the polyester bags were washed and dried as described for Experiment 2.

3. 3. 5 Sample Analysis

CT content was determined using the method of Terrill *et al.* (1992), as described by Yu *et al.* (1993). Extractable CT was extracted using a mixture of acetone/water/diethyl ether (4.7:2.0:3.3 v/v), followed by extraction of protein-bound CT using boiling sodium dodecyl sulphate containing 2-mercaptoethanol in 10 mM Tris/Chloride, adjusted to pH 8.0 with HCl (SDS solution). Fibre-bound CT was determined by boiling the residue remaining from protein extraction with butanol-HCl and SDS solution. CT concentration in each fraction was then determined by the butanol-HCl procedure (Porter *et al.* 1986). Free gossypol was determined using method Ba 7-58 of the American Oil Chemists Society (AOCS 1975). Total nitrogen (N) was determined by the Kjeldahl procedure, and crude protein calculated as total N × 6.25. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin were determined using the method of Robertson and Van Soest (1981). Dry matter was determined by drying at 100°C for 24 hours and oil content was determined by extraction with petroleum ether (Boiling point 40-60°C) for 8 hours.

In Experiment 2, the polyester bag residues remaining after total N analysis were pooled for each treatment at each time point (n=6 per treatment), for determination of *in situ* solubility of the 52 kDa and 48 kDa proteins in cottonseed kernel using the SDS-PAGE method of McNabb *et al.* (1994), as described by Yu *et al.* (1995a).

Between 100 and 1600 µg DM samples containing approximately 8 µg of protein from polyester-fibre bag residue were loaded in each well, and the electrophoresis was carried out for approximately 3 h at 80 V. The mini-gels (1.0 × 75 × 100 mm) consisted of a stacking gel approximately 22 mm high, layered over a separating gel. After SDS-PAGE, the gels were washed and then total soluble protein was visualised by staining with Fast Green FCF (0.1% Fast Green FCF; 40% methanol; 10% acetic acid) for 30 min. The gels were destained in 10% methanol; 7.5% acetic acid. The bands of the 48 kDa and 52 kDa proteins on the developed gels were scanned and quantified using imaging densitometry (Bio-Rad, Model GS-670 Imaging Densitometer, USA).

3. 3. 6 Calculation of Data and Statistical Analysis

Data from Experiment 1 were subjected to a one-way analysis of variance. Significant differences between the treatment groups were determined by the least significant difference (LSD) test (Steel and Torrie 1980).

In Experiment 2, the *in situ* DM digestion and N solubility rate in the rumen was calculated using the following equation (Ørskov and McDonald 1979).

$$Y = A+B\ (1-e^{-Ct}) \qquad (1)$$

where Y represents percent DM digestion at time (t) in hours spent in the rumen. The constants A, B and C represent, respectively, the instantly soluble fraction (A), the proportion digested in time t (B) and the digestion rate of the 'B' fraction (C). The constants A, B, C for each animal were calculated by using NLIN (non-linear regression) procedures (SAS 1985). Potential digestibility was calculated as $A+B$. Predicted rumen digestibility (P) was calculated from the equation of Ørskov and McDonald (1979).

$$P = A + [BC/(C+k)] \qquad (2)$$

where k is the rumen particulate dry matter fractional outflow rate, determined with sheep fed a similar lucerne hay to that used in the present investigation (0.033 h^{-1}; Domingue *et al.* 1991). The same procedures were used to quantify N solubility, with rumen fractional outflow rate being assumed to be 0.046 h^{-1} (Ørskov and McDonald 1979).

The significance of differences between means for C, $A+B$ and P was established using GLM (General Linear Models) procedures (SAS 1985), with the factors examined being level of hulls, PEG, animals, and the PEG × hulls and PEG × time interactions. If the hulls or the hulls × PEG interaction was significant ($p<0.05$), then the hulls and hulls × PEG effects were partitioned into linear and quadratic effects and their interactions with PEG.

In Experiment 3, the *in situ* DM digestion rate was calculated from the equation of Mead and Curnow (1983).

$$Y = (A+B)/[1+e^{(A-Ct)}] \qquad (3)$$

where Y represents DM digestion at time (t) in hours spent in the rumen, and the constants A, B and C have the same meaning as that described for equation (1). Predicted rumen digestibility (P) was also calculated using equation (2). The significance of differences between treatments for the constants C, $A+B$, and P was tested using one way analysis of variance.

3. 4 RESULTS

3. 4. 1 Experiment 1

In a mixture of 1 g cottonseed kernel with 200% hulls, 23% of total N was soluble in mineral buffer in the absence of PEG. This was increased to 30% at 2 mg PEG mg^{-1} CT and further addition of PEG did not result in further increases in soluble N, showing that two mg PEG mg^{-1} total CT was required to prevent or reverse binding of CT in hulls to cottonseed protein.

In the absence of hulls, 42% of the total N in cottonseed kernel was soluble in mineral buffer (Figure 3. 1). This progressively declined ($p<0.05$) as increasing quantities of hulls were added to the kernel. The addition of PEG increased N solubility ($p<0.05$) at each level of hull addition, although values did not increase to the same level as found for pure cottonseed kernel. In PEG treated flasks, N solubility still declined with increasing levels of hull addition.

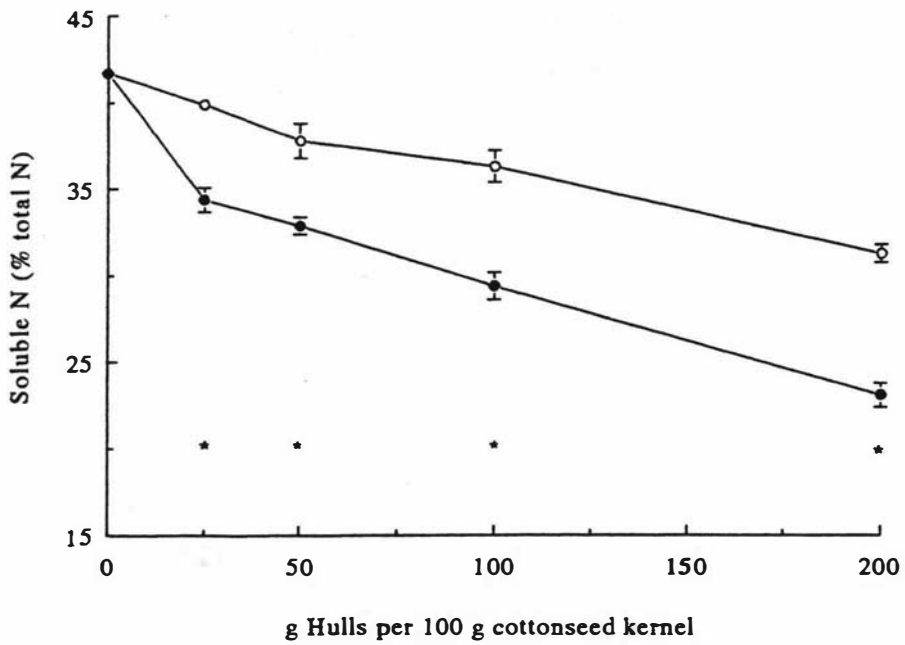

Fig 3. 1 Experiment 1b. The effect of adding cottonseed hulls and polyethylene glycol (PEG; MW 3,500) upon the *in vitro* nitrogen solubility of solvent extracted cottonseed kernel in phosphate mineral buffer (pH 7.0). Each point is the mean of 3 flasks per treatment. PEG was added at 2 mg mg^{-1} of total CT. ●, cottonseed kernel + hulls; ○, cottonseed kernel + hulls + PEG; I, standard error; *, $p\leq0.05$.

3. 4. 2 Experiment 2

Increasing rate of hull addition to cottonseed kernel (Table 3. 2) had no effect upon the *in situ* solubilization rate (constant *C*; 0.17% h^{-1}) of total N, but linearly decreased potential N solubility (constant *A+B*; $p<0.001$) and predicted rumen N solubility (*P*; $p<0.001$). PEG had no effect upon potential N solubility or predicted N solubility. The interactions among all treatments were not significant.

Table 3. 2 Experiment 2. Effect of adding cottonseed hulls upon rates of *in situ* N solubility of extracted cottonseed kernel in the rumen of sheep intra-ruminally infused with water or water containing polyethylene glycol (PEG; MW 3,500)

Constant	Solubilization rate C (% h^{-1})		Potential solubility A+B (%)		Predicted solubility P (%)	
PEG	-	+	-	+	-	+
Cottonseed kernel	0.17	0.16	99	99	86	87
Kernel+25% hulls	0.19	0.15	95	97	84	82
Kernel+50% hulls	0.15	0.18	94	94	82	81
Kernel+100% hulls	0.16	0.19	95	93	78	80
Kernel+150% hulls	0.16	0.18	89	90	76	76
Kernel+200% hulls	0.16	0.19	88	90	75	74
SEM[1]	0.019		1.2		1.0	
Statistical effects[2]						
Hulls-linear	NS		***		***	
-quadratic	NS		NS		NS	
PEG	NS		NS		NS	
Hulls × PEG	NS		NS		NS	

[1] Mean standard error (n=6).

[2] NS $p>0.05$; *** $p<0.001$.

Solubilization of the 52 and 48 kDa cottonseed kernel proteins was variable during the initial 12 h incubation in the rumen, but stable values were attained between 24 and 48 h of incubation, with the mean value over this period being referred to as maximum solubility. Averaged over all six treatment groups, approximately 61 and 70% of the 52 and 48 kDa proteins were instantly soluble in water (ie disappeared at 0 h of incubation), and 97 and 98% were solubilized during the initial 8 h of incubation, indicating that these storage proteins in cottonseed kernel were highly soluble in the rumen. Addition of hull and PEG had no effect upon the *in situ* maximum solubility of both the 52 and 48 kDa proteins (Table 3. 3).

Increasing rates of hull addition to cottonseed kernel progressively depressed *in situ* DM digestion rate (constant *C*), potential DM digestibility (constant *A+B*) and predicted rumen DM digestibility (*P*; p<0.001; Table 3. 4). These effects were best described by quadratic relationships (p<0.001), with increasing rate of hulls depressing these parameters by progressively smaller amounts. PEG had no effect upon digestion rate, but increased both potential rumen DM digestibility (p<0.01) and predicted rumen DM digestibility (p<0.01). The hulls × PEG interaction was significant (P<0.05) for predicted rumen DM digestibility, explained by PEG addition progressively increasing DM digestibility with increasing rate of hull addition but having no effect in the absence of hulls. Similar effects were apparent for potential rumen DM digestibility, but in this instance the hull × PEG interaction did not attain significance (p>0.05).

Table 3. 3 Experiment 2. Effect of adding cottonseed hulls upon the *in situ* maximum solubility[1] of the 52 and 48 kDa proteins in extracted cottonseed kernel in the rumen of sheep intra-ruminally infused with water or water containing polyethylene glycol (PEG; MW 3,350)

	52 kDa protein solubility (%)		48 kDa protein solubility (%)	
PEG	-	+	-	+
Cottonseed kernel	99.2	95.0	99.6	98.5
Kernel+25% hulls	97.3	99.9	97.6	99.8
Kernel +50% hulls	99.9	99.4	99.9	99.7
Kernel+100% hulls	98.3	99.7	97.2	99.5
Kernel+150% hulls	99.8	99.8	99.9	99.6
Kernel+200% hulls	99.7	99.4	99.7	99.6

[1] Average of three values for solubility measured at 24, 36 and 48 hours of incubation. Samples were pooled from sheep on each treatment at each sampling time, to give one value at each time for each treatment.

3. 4. 3 Experiment 3

In situ DM digestion (%) of cottonseed hulls in the rumen of sheep is shown in Figure 3. 2. The rate of *in situ* DM digestion was very slow for 12 h, and then progressively increased to 48 h. Although the *in situ* DM soluble component (constant *A*) was not affected by PEG (Table 3. 5), the *in situ* insoluble component (constant *B*; p<0.01), digestion rate (p<0.05), potential rumen digestibility (p<0.01) and predicted rumen digestibility (p<0.01) of DM were all significantly increased by PEG. Predicted rumen digestibility was increased by 7% units.

Table 3. 4 Experiment 2. Effect of adding cottonseed hulls upon rates of *in situ* DM digestion of extracted cottonseed kernel in the rumen of sheep intra-ruminally infused with water or water containing polyethylene glycol (PEG; MW 3,500)

	Digestion rate C (% h^{-1})		Potential digestibility A+B (%)		Predicted digestibility P (%)	
PEG	-	+	-	+	-	+
Cottonseed kernel	0.17	0.16	99	99	88	87
Kernel+25% hulls	0.15	0.12	86	89	75	75
Kernel+50% hulls	0.10	0.12	80	81	66	68
Kernel+100% hulls	0.09	0.08	72	74	55	58
Kernel+150% hulls	0.07	0.06	66	70	49	51
Kernel+200% hulls	0.06	0.06	64	67	45	48
SEM[1]	0.009		1.4		0.7	
Statistical effects[2]						
Hulls-linear	***		***		***	
-quadratic	**		***		***	
PEG	NS		**		**	
Hulls × PEG	NS		NS		*	

[1] Mean standard error (n=6).

[2] NS $p>0.05$; * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

Table 3. 5 Experiment 3. *In situ* DM digestibility of cottonseed hulls in the rumen of sheep intra-ruminally infused with water or water containing polyethylene glycol (PEG; MW 3,500)

Constant	Soluble component A (%)	Insoluble component B (%)	Digestion rate C (% h^{-1})	Potential digestibility A+B (%)	Predicted digestibility P (%)
Hulls	2.4	38.1	0.09	40.5	32.7
Hulls+PEG	2.6	44.4	0.12	47.1	39.6
SEM[1]	0.12	0.75	0.008	0.78	0.89
Significant level[2]	NS	**	*	**	**

[1] Mean standard error (n=6).

[2] NS $p>0.05$; * $p<0.05$; ** $p<0.01$.

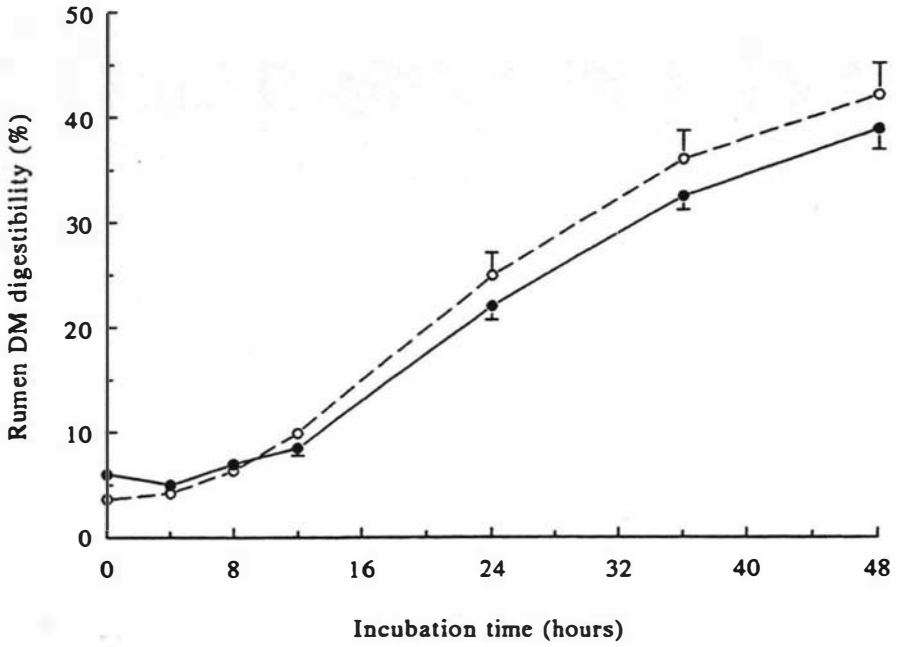

Fig 3. 2 Experiment 3. The *in situ* dry matter digestibility of ground cottonseed hulls in the rumen of sheep intra-ruminally infused with water or water containing polyethylene glycol (PEG; MW 3,500). Each point is the mean of 6 sheep per treatment. ●, hulls; ○ , hulls+PEG; I, standard error.

3. 5 DISCUSSION

In the absence of hulls, 42% of the total N in cottonseed kernel was soluble in mineral buffer, with rumen potential N solubility and predicted N solubility *in situ* being 99 and 86% respectively. These values show that the total N in unheated solvent extracted cottonseed kernel was highly soluble. Also, in the absence of CT, it is evident that PEG *per se* had no effect upon the solubility of total N in cottonseed.

Both the *in vitro* and *in situ* experiments showed that adding hulls depressed solubility of total N in cottonseed kernels in a linear relationship. However, the *in vitro*

PEG data indicated that part of the hull effect could be explained by the presence of CT in the hulls, but *in situ* data did not support this hypothesis. However, both the *in vitro* and *in situ* data indicated that a component in hulls other than CT depressed solubility of kernel total N. The mechanism for this is unknown. The magnitude of the depression in total N solubility caused by the addition of hulls was quite small, with 25% of hulls depressing predicted rumen N solubility by only 3.5% units. As whole cottonseed contains 33% hulls (Yu *et al.* 1993), it is likely that the hulls would have a minimal effect upon N solubility when whole cottonseed is fed to ruminant livestock. As maximum solubility of the 52 and 48 kDa kernel proteins was close to 100% and was not affected by the addition of either hulls or PEG, it seems that addition of hulls must have lowered the solubility of other kernel proteins.

Effects of CT upon solubility contrast with results of *in vitro* degradation studies (Yu *et al.* 1995a), where the addition of cottonseed hulls caused a consistent depression in degradability of the 52 and 48 kDa kernel proteins, with approximately 50% of the effect being due to CT in the hulls. One explanation may be that CT was more effective at reducing protein degradation rather than protein solubility, as also found by McNabb (1990) for CT in the forage of *Lotus pedunculatus*. For a range of protein concentrate meals, Stern and Satter (1984) found a low correlation (0.26) between protein solubility and protein degradation. Spencer *et al.* (1988) also suggested that the loss of N from synthetic-fibre bags suspended in the rumen measured the solubilization of plant protein, and that the rates of protein solubilization and degradation in the rumen were not necessarily similar. In the present study, the solubilization of N from polyester-fibre bags, particularly with the mixtures of cottonseed kernel and hulls, was not a good index of the effect of CT on protein degradation in the rumen.

Cottonseed hulls are a by-product of cottonseed processing, with cottonseed being cleaned and partially dehulled before kernels are crushed and subjected to oil extraction. Commercial cottonseed hulls consist primarily of the outer covering of cottonseed with lint fibres attached to the hulls (Harris 1991). Cottonseed hulls contain approximately 40 g kg^{-1} crude protein, 12 g kg^{-1} oil, 900 g kg^{-1} neutral detergent fibre and 32-65 g kg^{-1} total CT (80% in bound form; Yu *et al.* 1993), and are commonly used in cattle diets to supply fibre (Stern and Ziemer 1993). Tuncer *et al.* (1992) reported that the rumen DM digestibility of cottonseed hulls was 35%, and that urea and NaOH treatments did not improve its digestibility. Similar values were found for predicted rumen DM digestibility in the present study, but digestibility was increased by 7% units in the presence of PEG, indicating that bound CT was reducing the rumen digestion of cottonseed hulls. As cottonseed hulls are almost pure fibre, it seems that bound CT reduced fibre digestion. Similar but smaller effects were also seen in mixtures of hulls and kernel, with the response to PEG for rumen potential and

predicted DM digestibility increasing with increasing proportion of hulls in the mixture (Table 3. 4).

The CT molecule is unlikely to be digested in the rumen, and the increased loss in DM from cottonseed hulls in PEG sheep was most likely due to better cell wall digestion (Jones and Mangan 1977; Terrill *et al.* 1994). PEG may solubilize and remove some CT, but this can not be determined because of analytical problems in determining CT in digesta (Terrill *et al.* 1994). PEG is likely to remove extractable CT in cottonseed hulls (approximately 20% of total CT), but is unlikely to remove all bound CT and may complex with some *in situ*, thus reducing its binding to cell wall components and improving access for rumen micro-organisms.

This is the first study to show that bound CT have a major effect in reducing rumen digestion of fibre. It seems that bound CT in cottonseed hulls has more effect on rumen digestion of hull fibre than on rumen digestion of kernel protein, as Yu *et al.* (1995a) showed the effect of bound CT accounted for about half of the hull effect in reducing degradability. These effects can be explained by bound CT not being very mobile during rumen fermentation, and having a greater effect locally (ie. reducing fibre digestion in hulls) than transferring to another diet component (ie. reducing kernel protein solubility and degradation). Bound CT are also present in substantial concentrations in some tropical forage legumes (F S Jackson, personal communication) and in browse species (Semiadi *et al.* 1995), and may similarly restrict rumen fibre digestion in those species.

The presence of CT is part of the host plant resistance mechanism in cotton for defence against attack by insects and pathogenic micro-organisms (Fitt *et al.* 1992), and on this basis, it seems unrealistic to select for low CT concentration in cotton breeding programmes. Commercial CSM contains 150 to 300 g kg^{-1} hulls, and has a CT content of 8-16 g kg^{-1} (Yu *et al.* 1993). Heating, as employed during commercial CSM manufacturing, may have some effect upon the N solubilization. The present studies have been done with unheated solvent extracted cottonseed, so it would seem prudent to repeat some of these treatments involving heating. If bound CT in cottonseed still restricts rumen fibre digestion in hulls and protein digestion in the small intestine of monogastric animals, as found by Yu *et al.* (1995b), then it seems that the presence of hulls in commercial CSM should be reduced to the lowest possible levels.

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Chapter 4

EFFECT OF CONDENSED TANNIN IN COTTONSEED HULLS UPON THE *IN VITRO* DEGRADATION OF COTTONSEED KERNEL PROTEINS BY RUMEN MICRO-ORGANISMS

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4. 1 ABSTRACT

A 24 h *in vitro* rumen incubation procedure was developed to measure the effect of adding cottonseed hulls (containing condensed tannin; CT) upon degradation of the 52 and 48 kDa major seed storage proteins present in unheated solvent extracted cottonseed kernels (which does not contain CT). Proteins were fractionated using sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the protein bands quantified using imaging densitometry. Effects of CT were established by conducting incubations in the absence and presence of polyethylene glycol (PEG; molecular weight (MW) 3,500), which binds and inactivates CT. A set of *in vitro* experiments was conducted, in which degradation rate and potential degradability were measured. In the absence of hulls, potential degradability of both kernel proteins was very high (99% SE 0.5), with approximately 97% (SE 0.4) of this taking place within 8 h, and the addition of PEG did not effect degradation. Increasing rates of hull addition reduced the potential degradability of both kernel proteins in a linear manner, but did not affect degradation rate. Equal weights of hulls and kernel (i.e. 100% hulls) reduced potential degradability of the 52 and 48 kDa proteins by approximately 10% units. Addition of PEG increased degradation of both kernel proteins in incubations involving mixtures of hulls and kernels, with 2 mg PEG mg^{-1} CT being required to maximise this effect. However, the increase obtained accounted for only 50% of the depression in protein degradation caused by the addition of hulls. In all experiments, the 52 and 48 kDa proteins were similarly affected by the treatments applied. It was concluded that *in vitro* rumen degradability of the 52 and 48 kDa storage proteins in unheated solvent extracted cottonseed kernel was very high and close to 100%, and that this could be reduced by the addition of hulls in a linear relationship, with approximately half of the depression in potential degradability caused by hulls being due to effects of CT. However, even in the presence of hulls, the degradation of the 52 and 48 kDa proteins was still very high.

4. 2 INTRODUCTION

The extent to which dietary proteins are degraded in the rumen and the proportion which escapes degradation for subsequent hydrolysis and absorption in the intestine affects dietary nutritive value (Leng *et al.* 1977; Chalupa 1984; Ørskov 1991). Dietary proteins degraded in the rumen provide essential nutrients and co-factors for microbial growth or may increase the fermentable organic matter in the rumen (Preston and Leng 1987). Casein and Fraction 1 leaf protein (Rubisco) are degraded rapidly in the rumen; however, bovine serum albumin (BSA), ovalbumin, bovine submaxillary mucoprotein and sunflower albumin 8 proteins are relatively resistant to rumen degradation (Mangan 1972; Nugent and Mangan 1981; Nugent *et al.* 1983; McNabb *et al.* 1994).

Resistance of proteins to rumen degradation may occur naturally or can be produced by various chemical or physical treatments. Condensed tannin (CT) synthesised in some plants from the shikimic acid biochemical pathway (Swain 1979) can form complexes with dietary protein through hydrogen bonding, which are undegradable at rumen pH (5.5-7.0) but dissociate and release protein at abomasal pH (2.0-3.0; Jones and Mangan 1977). Many studies (see reviews by Mangan 1988; Barry 1989) have investigated the effects of CT in forages upon rumen protein digestion, but there is no information on the effects of CT upon rumen protein degradation of processed oil-seed meals. Cottonseed meal (CSM) is an important protein supplement for ruminants, and commercial CSM produced in Australia contains 700 to 850 g kg^{-1} solvent extracted cottonseed kernel (to remove the oil) and 150 to 300 g kg^{-1} hulls, and has a CT content of 8-16 g kg^{-1} (Yu *et al.* 1993). CT is present in the cottonseed hulls (32-65 g kg-1 DM) but not in kernels, and unlike forages, most CT in cottonseed hulls is bound to protein and fibre (approximately 80% of total CT) rather than being extractable in 70:30 acetone:water (Yu *et al.* 1993). Adding cottonseed hulls has been shown to reduce both *in vitro* and *in vivo* solubility of the total N in cottonseed kernels (Yu *et al.* 1995a).

Cottonseed kernels contain three major classes of seed storage proteins; 2S, 5S and 9S, in approximately equal amounts; 5S and 9S proteins are globulins, whilst the 2S proteins are albumins (Youle and Huang 1979). Storage proteins comprise about 70% of the total protein in cottonseed kernels, and are located within protein bodies (aleurone grains; Lui and Altschul 1967). The two principal storage proteins (α-globulin and β-globulin) have been identified in cottonseed kernel, and have molecular weights of 52,000 and 48,000 Da. They have similar solubilities, ultraviolet absorption spectra, and contain similar proportions of amino acid residues (Dure and Chlan 1981).

Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has been used to determine the relative rumen degradability of individual proteins from

lucerne, sunflower seed, soybean meal, lupin and rape seed incubated with rumen fluid both *in vivo* and *in vitro* (Nugent and Mangan 1981; Nugent *et al.* 1983; Spencer *et al.* 1988; Romagnolo *et al.* 1990; Cherney *et al.* 1992; McNabb *et al.* 1994). The objective of the present study was to measure rumen degradability of the two major storage proteins (52 and 48 kDa) in cottonseed kernels, to measure the effect of adding cottonseed hulls upon the degradation of kernel proteins, and to assess the extent to which this effect that was due to CT. In this paper, degradation is defined as the rate of degradation of the 52 and 48 kDa proteins from cottonseed kernels incubated with rumen fluid, with both proteins being fractionated by SDS-PAGE and quantified using imaging densitometry.

4. 3 MATERIALS AND METHODS

4. 3. 1 Experiment Design

Four *in vitro* experiments were conducted, with each treatment being done in duplicate, to measure the degradation of the 52 and 48 kDa proteins from cottonseed kernels during incubation with rumen fluid. All experiments were done in the presence or absence of polyethylene glycol (PEG; molecular weight (MW) 3,500, Union Carbide, Danbury, CT, USA). The PEG binds and inactivates CT (Jones and Mangan 1977) so that the effects of CT can be assessed by comparing control flasks (CT acting) with PEG flasks (CT inactivated).

In Experiment 1, the effect of extracting total protein from kernels, protease activity and energy addition (cellobiose; a readily fermentable sugar used by a wide range of micro-organisms) were determined to develop an *in vitro* rumen incubation procedure which was used in all subsequent experiments. The cottonseed kernels or total protein extracted from cottonseed kernels, incubated with either untreated rumen fluid or autoclaved rumen fluid, with or without two levels of added cellobiose (150 and 375 mg flask^{-1}) were compared in this experiment.

Experiment 2 compared the protein degrading capacity of rumen fluid from sheep fed a basal diet of meadow hay supplemented with iso-nitrogenous amounts of either lucerne chaff or commercial cottonseed kernel, and also the effects of adding hulls and PEG to cottonseed kernels. The treatments for Experiment 2 were cottonseed kernel plus either 0 or 100% hulls (i.e. 1000 mg kernel + either 0 or 1000 mg hulls), with or without PEG (1.5 mg mg^{-1} total CT), incubated with rumen fluid from sheep supplemented with either lucerne chaff or commercial cottonseed kernel.

In Experiment 3, the effect of adding cottonseed hulls (CT-containing) on degradation of cottonseed kernel proteins was investigated. The treatments for this

experiment were addition of either 0, 25, 50, 100, 150, or 200% hulls to cottonseed kernel, incubated with rumen fluid from sheep supplemented with commercial cottonseed kernel.

Experiment 4 investigated the effect of adding PEG upon the degradation of cottonseed kernel proteins, in the presence or absence of hulls. The treatments for Experiment 4a were cottonseed kernel plus either 0, 100 or 250 mg PEG and for Experiment 4b were kernel+100% hulls plus several levels of PEG (0, 1, 2, 3, 4, and 5 mg mg^{-1} total CT), incubated with rumen fluid of sheep supplemented with commercial cottonseed kernel.

4. 3. 2 Preparation of Cottonseed Samples

Delinted cottonseed (var. Siokra L22) supplied by Cotton Seed Distributors Ltd, Wee Waa, NSW, Australia was cracked using a Crushing-Mill (AB Thorell and Persson, Uppsala, Sweden), and then separated into kernels and hulls using an air-blow technique at the Seed Technology Centre, Massey University. Separated kernels were freeze-dried for 48 h, ground, and the oil and gossypol extracted using a modification of the Pons and Eaves (1967) procedure, as described by Yu *et al.* (1995a). The extracted cottonseed kernel and untreated hulls then were ground to pass through a 1 mm diameter sieve and were stored at -20°C. The chemical composition of extracted cottonseed kernel and hulls is shown in Table 4. 1.

Experiment 1 used endosperm total protein which was prepared by extracting cottonseed kernels overnight at room temperature in a solution of 2% sodium-dodecyl-sulphate (SDS), 1% mercaptoethanol, and 50 mM Tris(hydroxymethyl)-HCl (pH 8.3) according to the method of Dure and Chlan (1981). The resulting extract contained 99% of the cotyledon protein and 59 mg ml-1 crude protein.

4. 3. 3 Preparation of Rumen Fluid

Four wether sheep (two in each treatment) were used for collection of rumen fluid for *in vitro* incubation. They were fitted with a rumen cannula (55 mm ID) and were maintained on a basal diet of 800 g d^{-1} meadow hay (890 g kg^{-1} DM, 20 g kg^{-1} N) supplemented with either 250 g d^{-1} lucerne chaff (860 g kg^{-1} DM, 30 g kg^{-1} N) or 125 g d^{-1} commercial cottonseed kernels (950 g kg^{-1} DM, 55 g kg^{-1} N, 2.3 g kg^{-1} total CT), which were unextracted and contained approximately 5% hulls (Yu *et al.* 1993). All feed was offered at hourly intervals, from overhead belt-feeders, for at least 2 weeks prior to each experiment commencing. Rumen fluid was collected at 08:00 h after overnight fasting, and quickly strained through muslin cloth into a Dewar flask flushed

with CO_2 gas. For each experiment rumen fluid was collected and pooled from two sheep fed the same diet, then maintained at 39°C under an atmosphere of CO_2, and used immediately for *in vitro* incubations.

Table 4. 1 Chemical composition[1] (g kg^{-1} DM) of the cottonseed components added to *in vitro* incubations with rumen fluid

	Cottonseed Kernel[2]	Cottonseed Hulls
Dry matter	909	911
Crude protein (N×6.25)	537	35
Oil	138	10
Neutral detergent fibre	89	886
Acid detergent fibre	37	624
Lignin	27	209
Free gossypol	0.8	0.2
Condensed tannin:		
Extractable	0	13
Protein-bound	0	29
Fibre-bound	0	10
Total (calculated)	0	52

[1] Mean of duplicate determinations.

[2] Cottonseed kernel was extracted using hexane and acetone/water to remove oil and gossypol according to the method of Yu *et al.* (1995a).

4. 3. 4 *In Vitro* Incubation with Rumen Fluid

Duplicate *in vitro* incubations were performed using the method described by McNabb *et al.* (1994). Freshly prepared artificial saliva (60 ml, pH 6.8; McDougall 1948), which was saturated with CO_2 gas and maintained at 39°C, and rumen fluid (15 ml), prepared as described previously, were added to each volumetric flask (250 ml). Each flask was then flushed with CO_2 and cottonseed kernel, hulls and PEG added as shown in Table 4. 2 for each experiment. Each flask was fitted with a Bunsen valve and shaken (90 min^{-1}) at 39°C for 24 hours.

Aliquots (475 µl) were removed from each flask after 0, 0.5, 1, 2, 4, 8, 12, 16 and 24 h of incubation, and added to Eppendorf tubes containing 25 µl of 10% SDS, to prevent protein precipitating out of solution upon freezing and subsequent thawing. All samples were frozen immediately, stored at -20°C and used for protein analysis. The flasks were re-flushed with CO_2 after each sampling.

Table 4. 2 Sources of rumen fluid, and the quantities (mg flask^{-1}) of cellobiose, cottonseed kernel, cottonseed hulls and polyethylene glycol (PEG; MW 3,500) added in each of the four *in vitro* experiments

Diet of sheep	Rumen fluid	Cellobiose	Cottonseed kernel[1]	Extracted protein[2]	Cottonseed hulls	PEG
Experiment 1A:						
ML[3]	autoclaved	-	-	177	-	-
ML	autoclaved	-	320	-	-	-
ML	non-autoclaved	-	-	177	-	-
ML	non-autoclaved	-	320	-	-	-
Experiment 1B:						
ML	non-autoclaved	0	-	177	-	-
ML	non-autoclaved	150	-	177	-	-
ML	non-autoclaved	375	-	177	-	-
ML	non-autoclaved	0	1000	-	-	-
ML	non-autoclaved	150	1000	-	-	-
ML	non-autoclaved	375	1000	-	-	-
Experiment 2:						
ML	non-autoclaved	150	1000	-	0	0
ML	non-autoclaved	150	1000	-	0	80
ML	non-autoclaved	150	1000	-	1000	0
ML	non-autoclaved	150	1000	-	1000	80
MC[4]	non-autoclaved	150	1000	-	0	0
MC	non-autoclaved	150	1000	-	0	80
MC	non-autoclaved	150	1000	-	1000	0
MC	non-autoclaved	150	1000	-	1000	80
Experiment 3:						
MC	non-autoclaved	150	1000	-	0	-
MC	non-autoclaved	150	1000	-	250	-
MC	non-autoclaved	150	1000	-	500	-
MC	non-autoclaved	150	1000	-	1000	-
MC	non-autoclaved	150	1000	-	1500	-
MC	non-autoclaved	150	1000	-	2000	-
Experiment 4A:						
MC	non-autoclaved	150	1000	-	0	0
MC	non-autoclaved	150	1000	-	0	150
MC	non-autoclaved	150	1000	-	0	250
Experiment 4B:						
MC	non-autoclaved	150	1000	-	1000	0
MC	non-autoclaved	150	1000	-	1000	50
MC	non-autoclaved	150	1000	-	1000	100
MC	non-autoclaved	150	1000	-	1000	150
MC	non-autoclaved	150	1000	-	1000	200
MC	non-autoclaved	150	1000	-	1000	250

[1] Cottonseed kernel was extracted using hexane and acetone/water to remove oil and gossypol according to the method of Yu *et al.* (1995a).

[2] Protein was extracted from oil and gossypol-free cottonseed kernel using a solution of 2% SDS, 1% mercaptoethanol, and 50 mM Tris-HCl, pH 8.3 using the method of Dure and Chlan (1981).

[3] ML; sheep fed on 800 g d^{-1} meadow hay and 250 g d^{-1} lucerne chaff (30 g N kg^{-1}).

[4] MC; sheep fed on 800 g d^{-1} meadow hay and 125 g d^{-1} commercial cottonseed kernel (55 g N kg^{-1}).

In Experiment 1 gas production was measured, using the method described by Waghorn and Stafford (1993), in order to investigate the optimum growth conditions for rumen micro-organisms. Each flask was connected to a manometric measuring device which enabled gas volumes to be measured at atmospheric pressure, with gas production recorded at 0.5, 1, 2, 3, 4, 6, 8, 12, 16, and 24 hours.

4. 3. 5 Protein Analysis by SDS-PAGE

Proteins in samples taken from *in vitro* incubation with rumen fluid were resuspended in protein digestion buffer (64 mM Tris-HCl, pH 6.8 and (g l^{-1}) 100 glycerol, 20 SDS, 50 2-β-mercaptoethanol, 0.05 bromophenol blue) and fractionated by SDS-PAGE using a procedure described by McNabb *et al.* (1994). All samples were heated at 95°C for 5 min to denature protein and dissociate CT-protein complexes. Approximately 30 µg of protein were loaded in each well, and electrophoresis was carried out at constant current of 80 V for approximately 3 h. The mini-gels (0.75 × 75 × 100 mm; Bio-Rad, Hercules, CA, USA) consisted of a stacking gel (12.5 mM Tris-HCl, pH 6.8; plus (g l^{-1}): 38.9 acrylamide, 1.1 bisacrylamide, 1 SDS, 1 ammonium persulphate, 1 tetramethyl-ethylenediamine) approximately 22 mm high, layered over a separating gel which contained 37.5 mM Tris-HCl, pH 8.8 and (g l^{-1}) 155.7 acrylamide, 4.3 bisacrylamide, 1 SDS, 0.5 ammonium persulphate, and 0.5 tetramethyl-ethylenediamine.

After SDS-PAGE, the gels were washed in 40% methanol; 10% acetic acid and total soluble protein was visualised by staining with Coomassie Brilliant Blue R-250 (5 g l^{-1} ethanol-acetic acid (40:25, v/v)) for 30 min and de-stained in 10% methanol; 7.5% acetic acid to detect protein bands. Godshall (1983) demonstrated that tannic acid interfered with the staining of protein by Coomassie Brilliant Blue G-250, however tannic acid did not interfere with the staining of protein by Coomassie Brilliant Blue R-250. Developed Coomassie Blue stained gels were quantified by imaging densitometry (Bio-Rad, Model GS-670 Imaging Densitometer, USA). The data were processed using image analysis software (Bio-Rad Molecular Analyst™/CP imaging analysis software, USA).

4. 3. 6 Other Chemical Methods

Samples of cottonseed kernel and hulls were analysed in duplicate for total nitrogen using the Kjeldahl procedure, and crude protein calculated as total N × 6.25. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin were determined using the method of Robertson and Van Soest (1981). Dry matter was determined by drying at 100°C for 24 hours and oil content was determined by extraction with petroleum ether (Boiling point (BPt) 40-60°C) for 8 hours. Free gossypol was determined using method

Ba 7-58 of the American Oil Chemists Society (AOCS; 1975), and extractable, protein-bound and fibre-bound CT was determined using the method of Terrill *et al.* (1992), as described by Yu *et al.* (1993).

4. 3. 7 Calculation of Data and Statistical Analyses

The disappearance of each protein, represented by change in density of the protein bands on the gels, were plotted (log scale) against time of incubation.

The *in vitro* degradation of each cottonseed kernel protein during incubation with rumen fluid was calculated by fitting the following regression equation (Ørskov and McDonald 1979),

$$Y = A+B\ (1-e^{-Ct}) \qquad (1)$$

Percent degradation of the 52 or 48 kDa proteins is represented by (Y), whilst (t) represents the time in hours of *in vitro* incubation. The constants A, B and C represent, respectively, the instantly soluble fraction at time 0 (A), the proportion degraded during time (t; B) and the rate of degradation of the 'B' fraction (C). ($A+B$) represents potential degradability. The constants A, B, C for each incubated flask were calculated by using non-linear regression (NLIN) procedures (SAS 1985).

The significance of differences among treatment means in each experiment was determined for the constants $A+B$ and C, and also for degradability after 8 h incubation. using general linear models (GLM) procedures (SAS 1985). In Experiments 3 and 4, if the main factor was significant ($p<0.05$), then the factor was partitioned into linear and quadratic effects.

4. 4 RESULTS

4. 4. 1 Experiment 1

There was no degradation of the 52 and 48 kDa proteins in either kernels or extracted kernel proteins when autoclaved rumen fluid was used, showing that there was no protease activity which could be attributed to either of these cottonseed preparations (Figure 4. 1). Both the extracted or non-extracted 52 and 48 kDa proteins of cottonseed kernel were degraded during *in vitro* incubation with rumen fluid that was not autoclaved (Figure 4. 1), with degradation of extracted proteins being much faster than that of non-extracted kernel proteins. The extracted proteins were almost completely degraded after 8 h incubation, whereas non-extracted kernel proteins were completely degraded after 24 h.

Adding supplementary energy (cellobiose) to rumen fluid had no effect on *in vitro* degradation of non-extracted kernel proteins, but increased the rate of degradation of extracted kernel proteins (Figure 4. 2). Adding cellobiose increased total gas production during the 24 h incubation, with rates of gas production for incubations involving extracted kernel proteins or non-extracted kernels plus 0, 150, and 375 mg cellobiose being -0.4, 1.2 and 3.7 vs 3.5, 5.6 and 8.7 ml h^{-1}, respectively. Maximum rates of gas production occurred during the first 4 h of incubation, followed by a rapid reduction in gas production after 8 h incubation in all treatments. However, adding cellobiose reduced the decline in gas production during the 8-24 h period.

In all subsequent experiments, 1000 mg non-protein extracted kernel and 150 mg cellobiose were incubated for 24 h with rumen fluid that was not autoclaved. Extracted kernel proteins were not used in Experiments 2, 3 and 4 because extraction of protein using SDS denatures proteins which would alter their rate of degradation. Cottonseed kernel which had been extracted for oil and gossypol more closely resembles the form of cottonseed used as a supplement for animals. Cellobiose was also added in order to maintain fermentation activity and gas production of rumen micro-organisms.

4. 4. 2 Repeatability of the experiments

Degradation of the 48 kDa protein in incubations involving cottonseed kernel only and kernel+100% hulls in Experiments 2, 3 and 4 is shown in Figure 4. 3. These two treatments were selected, because they were common to all three experiments. Repeatability of the degradation of the 48 kDa protein in both treatments was very high, indicating that the technique used was constant and reliable. The data fitted well to Equation 1, with the regression accounting for over 99% of the total sums of squares in all these experiments.

4. 4 .3 Experiment 2

Degradation rate (constant C) of both the 48 kDa protein ($p<0.01$) and of the 52 kDa protein ($p<0.05$) was greater when using rumen fluid from sheep supplemented with commercial cottonseed kernel than with lucerne hay (Table 4. 3). The effects of source of rumen fluid upon potential degradability (constant $A+B$) and degradability at 8 h were smaller in magnitude and not always consistent.

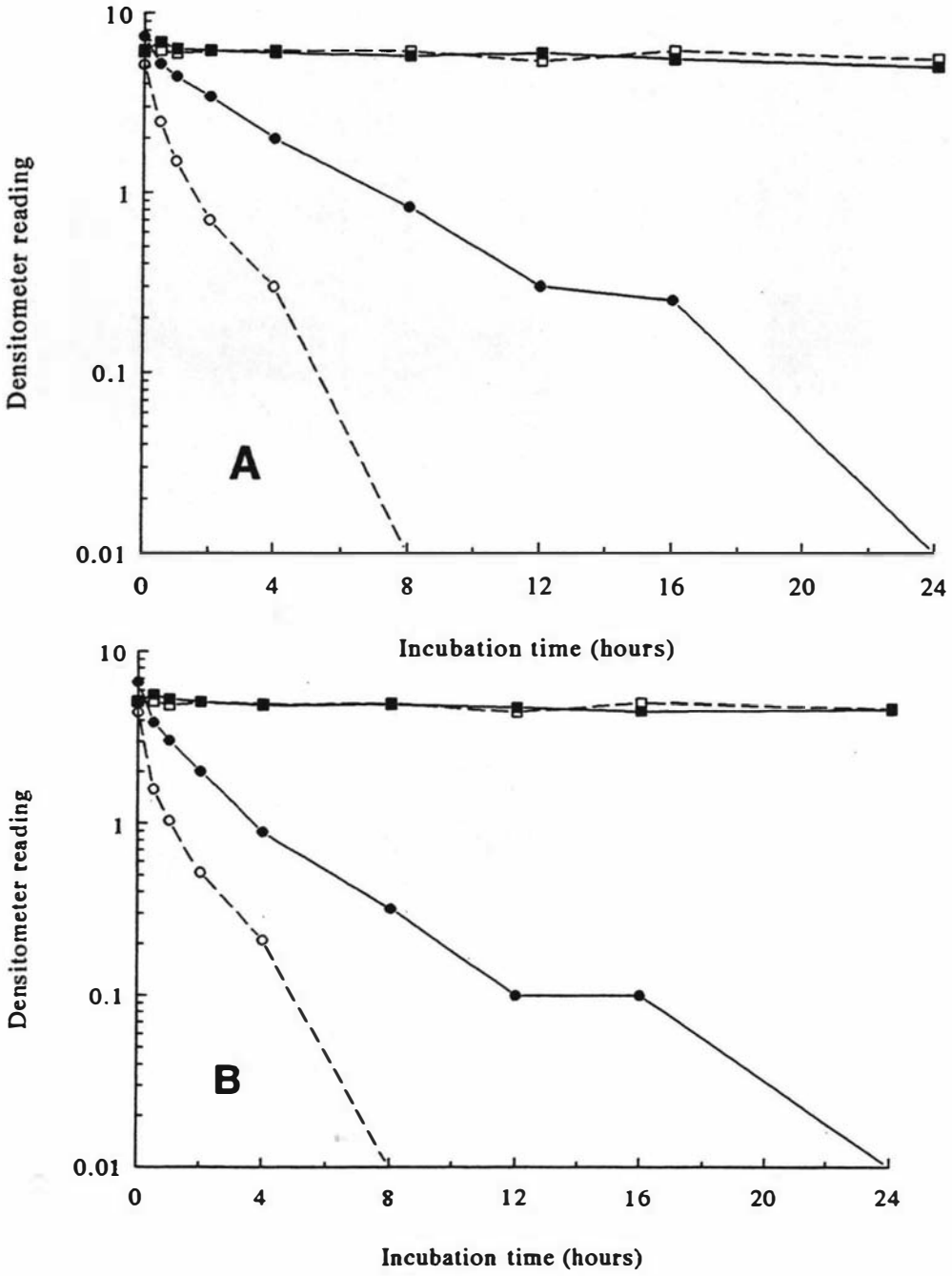

Fig. 4. 1 Experiment 1a. Degradation of the extracted or non-extracted 48-kDa protein (A) and 52-kDa protein (B) of cottonseed kernel in an *in vitro* incubation with rumen fluid or autoclaved rumen fluid. Rumen fluid was collected from sheep fed on 800 g d^{-1} meadow hay and 250 g d^{-1} lucerne chaff. Fifteen ml of strained rumen fluid, 60 ml of artificial saliva and either 320 mg of ground cottonseed kernel or 177 mg extracted total kernel protein were incubated at 39°C for 24 h. ● kernel incubated with rumen fluid; ○ extracted protein incubated with rumen fluid; ■ kernel incubated with autoclaved rumen fluid; □ extracted protein incubated with autoclaved rumen fluid. Each point is the mean of duplicate determinations.

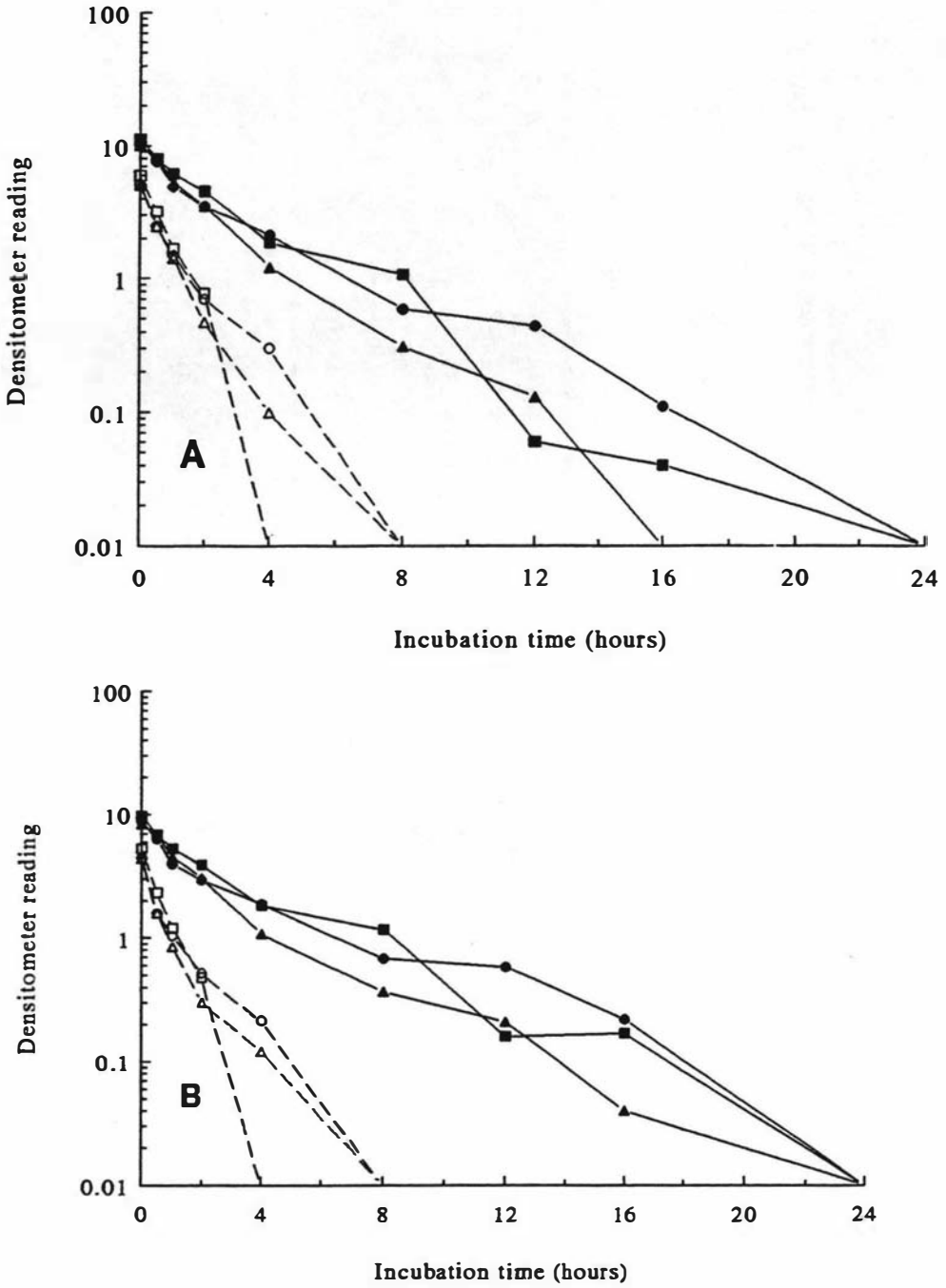

Fig. 4. 2 Experiment 1b. Degradation of the extracted or non-extracted 48-kDa protein (A) and 52-kDa protein (B) of cottonseed kernel during the *in vitro* incubation in rumen fluid supplemented with energy (cellobiose). Rumen fluid was collected from sheep fed on 800 g d^{-1} meadow hay and 250 g d^{-1} lucerne chaff. Fifteen ml of strained rumen fluid, 60 ml of artificial saliva and 1 g of cottonseed kernel supplemented with or without cellobiose were incubated at 39°C for 24 h. ● kernel; ▲ kernel+150 mg cellobiose; ■ kernel+375 mg cellobiose. ○ extracted protein; △ extracted protein+150 mg cellobiose; □ extracted protein+375 mg cellobiose. Each point is the mean of duplicate determinations.

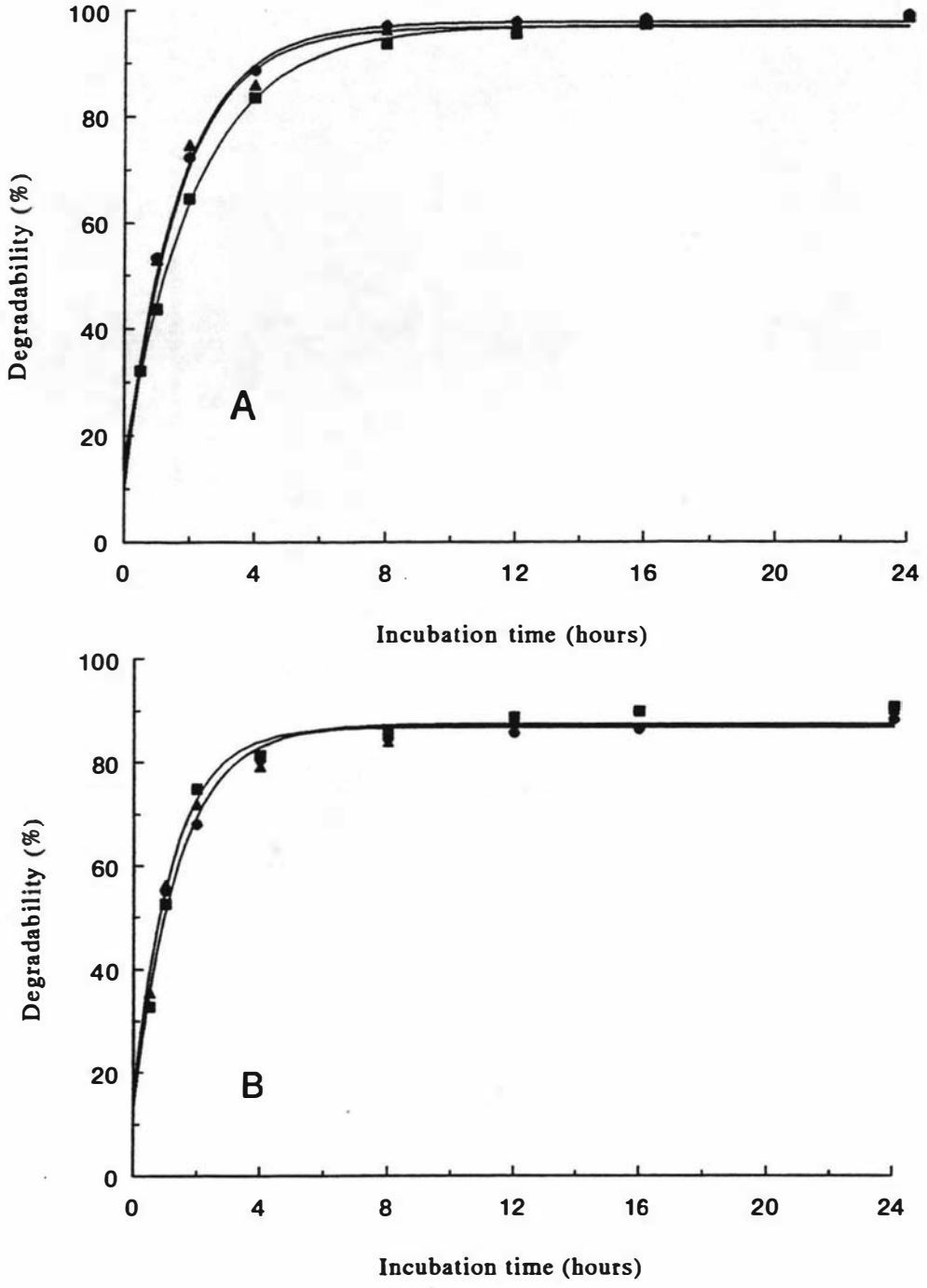

Fig. 4. 3 Experiment 2, 3 and 4. Repeatibility of degradation of the 48-kDa protein from cottonseed kernels only (A) and kernels + 100% hulls (B) during the *in vitro* incubation with rumen fluid. Protein degradation (Y;%) expressed as a percentage of that present at t=0, was fitted to the equation (Ørskov and McDonald 1979): $Y = A + B\,(1 - e^{-Ct})$. Rumen fluid was collected from sheep fed on 800 g d^{-1} meadow hay and 125 g d^{-1} cottonseed kernel. Fifteen ml of strained rumen fluid, 60 ml of artificial saliva, 150 mg cellobiose and 1 g of cottonseed kernel were incubated at 39°C for 24 h. ● Experiment 2; ▲ Experiment 3; ■ Experiment 4. Each point is the mean of duplicate determinations.

Adding cottonseed hulls significantly reduced potential degradability and degradability after 8 h of both the 52 and 48 kDa proteins ($p<0.01$; Table 4. 3). There was a significant interaction ($p<0.05$) between PEG and hulls for potential degradability of both proteins, explained by PEG addition to remove CT increasing degradability in the presence of hulls but not in the absence of hulls. This trend was apparent in all four experimental groups in Table 3, and whilst PEG addition increased potential degradability in the presence of 100% hulls, in all instances the values never attained the potential degradability of kernel alone. The same trends were apparent for degradability at 8 h. Degradation rate was not affected by addition of either hulls (0.49 vs 0.51, 0.51 vs 0.49% h^{-1} for the 48 and 52 kDa proteins) or PEG (0.53 vs 0.47, 0.53 vs 0.48% h^{-1} for the 48 and 52 kDa proteins).

Based upon the results of Experiment 2, rumen fluid from sheep fed meadow hay supplemented with commercial cottonseed kernel (not lucerne hay) was used in all future experiments.

4. 4. 4 Experiment 3

Increasing rates of hull addition to cottonseed kernel linearly depressed *in vitro* potential degradability ($p<0.001$) and degradability after 8 h ($p<0.001$), with the effects being equally apparent for both the 52 and 48 kDa proteins (Table 4. 4). Degradation rate was not affected by increasing rates of hull addition. Averaged over all six treatment groups, approximately 98% of the potentially degradable 52 and 48 kDa proteins disappeared during the initial 8 h of incubation, indicating that the storage proteins (52 and 48 kDa) of cottonseed kernel were not resistant to degradation.

4. 4. 5 Experiment 4

Addition of PEG to incubations of cottonseed kernel did not affect *in vitro* degradation rate, potential degradability and degradability after 8 h of either the 52 or 48 kDa proteins (Table 4. 5). Degradability of the 52 and 48 kDa proteins after 8 h was 98% of total potential degradability for each protein.

Addition of different levels of PEG to a mixture of kernel+100% hulls increased degradation rate of both proteins in a cubic relationship, with maximum values at 2 mg PEG mg^{-1} CT (Table 4. 5). PEG addition increased potential degradability of both proteins in a quadratic relationship ($p<0.01$). Degradability of both proteins at 8 h incubation tended to increase linearly when PEG was added. Most of the increase in potential degradability occurred with the first level of PEG addition (1 mg mg^{-1} CT), whilst all of the increase was accomplished by the second level of addition (2 mg mg^{-1}

Table 4. 3 Experiment 2. Effect of adding hulls and/or polyethylene glycol (PEG; MW 3,500) upon the degradation[1] of cottonseed kernel (CSK) proteins during *in vitro* incubation with rumen fluid (RF)[2]

	48-kDa protein			52-kDa protein		
Treatment	Degradation rate C (% h^{-1})	Potential degradability A+B (%)	Degradability at 8 h. (%)	Degradation rate C (% h^{-1})	Potential degradability A+B (%)	Degradability at 8 h. (%)
Incubation with Lucerne RF[3]						
Kernel	0.36	101	96	0.36	101	97
Kernel+80mg PEG	0.28	102	96	0.31	102	97
Kernel+100% hulls	0.36	87	81	0.39	88	83
Kernel+100% hulls+80mg PEG (1.5 mg mg^{-1} CT)	0.19	96	87	0.18	96	87
Incubation with CSK RF[4]						
Kernel	0.59	98	97	0.59	98	98
Kernel+80mg PEG	0.74	98	97	0.80	99	98
Kernel+100% hulls	0.82	86	84	0.78	88	87
Kernel+100% hulls+80mg PEG (1.5 mg mg^{-1} CT)	0.67	90	88	0.62	91	91
SEM[5]	0.09	1.7	2.0	0.09	1.5	1.4
Statistical effects						
RF	**	*	NS	*	*	p=0.09
Hulls	NS	**	**	NS	**	**
PEG	NS	*	NS	NS	*	NS
Hulls×PEG	NS	*	p=0.12	NS	*	p=0.16

[1] Protein degradation (Y;%) expressed as a percentage of that present at t=0, was fitted to the equation (Ørskov and McDonald 1979)

$$Y = A + B\,(1 - e^{-Ct})$$

where A,B and C are constants, and t is the time (h) of incubation with rumen fluid. Mean of duplicate determinations.

[2] Fifteen ml of rumen fluid, 60 ml of artificial saliva, 150 mg of cellobiose (Sigma) and 1 g of cottonseed kernel with or without added hulls and/or PEG were incubated at 39°C for 24 h.

[3] RF was collected from sheep fed on 800 g d^{-1} meadow hay (890 g DM kg^{-1}) and 250 g d^{-1} lucerne chaff (889 g DM kg^{-1}, 30 g N kg^{-1}).

[4] RF was collected from sheep fed on 800 g d^{-1} meadow hay (890 g DM kg^{-1}) and 125 g d^{-1} commercial cottonseed kernel (946 g DM kg^{-1}, 55 g N kg^{-1}).

[5] Mean standard error. NS $p>0.05$; * $p<0.05$; ** $p<0.01$.

Table 4. 4 Experiment 3. Effect of adding hulls upon the degradation[1] of cottonseed kernel proteins during *in vitro* incubation with rumen fluid[2]

	48-kDa protein			52-kDa protein		
Treatment	Degradation rate C (% h^{-1})	Potential degradability A+B (%)	Degradability at 8 h. (%)	Degradation rate C (% h^{-1})	Potential degradability A+B (%)	Degradability at 8 h. (%)
Kernel	0.71	97	97	0.71	98	98
Kernel+25% hulls	0.61	95	92	0.71	97	95
Kernel+50% hulls	0.53	95	93	0.52	97	94
Kernel+100% hulls	0.60	89	86	0.49	90	87
Kernel+150% hulls	0.91	82	82	0.78	82	82
Kernel+200% hulls	0.53	72	69	0.50	74	70
SEM[3]	0.10	2.9	3.5	0.08	2.7	2.8
Statistical effects						
Hulls-linear	NS	***	***	NS	***	***
-quadratic	NS	NS	NS	NS	NS	NS

1 Protein degradation (Y;%) expressed as a percentage of that present at t=0, was fitted to the equation (Ørskov and McDonald 1979)

$$Y = A + B\,(1 - e^{-Ct})$$

where A,B and C are constants, and t is the time (h) of incubation with rumen fluid. Mean of duplicate determinations.

2 Fifteen ml of rumen fluid, 60 ml of artificial saliva, 150 mg of cellobiose (Sigma) and 1 g of cottonseed kernel with or without added hulls were incubated at 39°C for 24 h. Rumen fluid was collected from sheep fed on 800 g d^{-1} meadow hay (890 g DM kg^{-1}) and 125 g d^{-1} commercial cottonseed kernel (946 g DM kg^{-1}, 55 g N kg^{-1}).

3 Mean standard error. NS $p>0.05$; *** $p<0.001$.

Table 4. 5 Experiment 4. Effect of adding cottonseed hulls and/or polyethylene glycol (PEG; MW 3,500) upon the degradation[1] of cottonseed kernel proteins during *in vitro* incubation with rumen fluid[2]

	48-kDa protein			52-kDa protein		
Treatment	Degradation rate C (% h^{-1})	Potential degradability A+B (%)	Degradability at 8 h. (%)	Degradation rate C (% h^{-1})	Potential degradability A+B (%)	Degradability at 8 h. (%)
Experiment 4A:						
Kernel	0.45	97	94	0.52	99	97
Kernel+150mg PEG	0.58	96	93	0.64	98	97
Kernel+250mg PEG	0.46	98	96	0.51	99	98
SEM[3]	0.04	0.5	1.8	0.04	0.6	1.8
Significance level	NS	NS	NS	NS	NS	NS
Experiment 4B:						
Kernel+100% hulls	0.87	89	89	0.73	91	91
Kernel+100% hulls+50mg PEG (1 mg mg^{-1} CT)	0.79	93	93	0.70	94	94
Kernel+100% hulls+100mg PEG (2 mg mg^{-1} CT)	1.03	94	93	1.05	95	95
Kernel+100% hulls+150mg PEG (3 mg mg^{-1} CT)	0.72	93	90	0.68	95	93
Kernel+100% hulls+200mg PEG (4 mg mg^{-1} CT)	0.66	93	90	0.67	95	92
Kernel+100% hulls+250mg PEG (5 mg mg^{-1} CT)	0.81	92	93	0.81	94	94
SEM[3]	0.03	0.6	1.1	0.06	0.7	1.1
Statistical effects						
PEG-linear	NS	*	p=0.09	NS	*	*
-quadratic	NS	**	NS	NS	**	NS
-cubic	**	NS	NS	*	NS	NS

[1] Protein degradation (Y;%) expressed as a percentage of that present at t=0, was fitted to the equation (Ørskov and McDonald 1979)

$$Y = A + B\,(1 - e^{-Ct})$$

where A, B and C are constants, and t is the time (h) of incubation with rumen fluid. Mean of duplicate determinations.

[2] Fifteen ml of rumen fluid, 60 ml of artificial saliva, 150 mg of cellobiose (Sigma) and both 1 g of cottonseed kernel and/or hulls with or without added PEG were incubated at 39°C for 24 h. Rumen fluid was collected from sheep fed on 800 g d^{-1} meadow hay (890 g DM kg^{-1}) and 125 g d^{-1} commercial cottonseed kernel (946 g DM kg^{-1}, 55 g N kg^{-1}).

[3] Mean standard error. NS $p>0.05$; * $p<0.05$; ** $p<0.01$.

CT). However, as in Experiment 2, the maximum increase in potential degradability induced by PEG addition was still less than potential degradability of kernel proteins in the absence of hulls.

4. 5 DISCUSSION

The principal findings of this study were that the *in vitro* rumen degradability of the 52 and 48 kDa storage proteins in unheated solvent extracted cottonseed kernel was very high, and that this could be reduced by the addition of hulls in a linear relationship, with approximately half of the depression in degradability caused by hulls being due to effects of CT. However, even in the presence of hulls, degradation of the 52 and 48 kDa proteins was still high.

The present results demonstrate that the *in vitro* incubation procedure, followed by separation of individual proteins using SDS-PAGE and their quantification by imaging densitometry can be successfully used to measure the degradation of individual storage proteins (MW 48 and 52 kDa) present in unheated solvent extracted cottonseed kernels. The application of SDS-PAGE was possible because the proteins in rumen fluid from sheep fed meadow hay plus either lucerne chaff or commercial cottonseed kernel, or pelleted lucerne hay (Spencer *et al.* 1988; McNabb *et al.* 1994) are heterogeneous and present in very low concentrations; therefore they do not interfere with the detection of added proteins. The SDS-PAGE procedure was standardised following the results of the initial experiment, and key factors adopted were the use of rumen fluid inoculum from sheep supplemented with commercial cottonseed kernels, the addition of cellobiose as an energy source to maintain fermentative activity, and the use of cottonseed kernel instead of extracted protein as the protein source. When the technique was standardised in this way, a high degree of repeatability was obtained, as shown in Figure 4. 3.

The rate of breakdown of any given protein in the rumen can vary with the animal's diet (Spencer *et al.* 1988). A change of diet from hay plus crushed oats to fresh lucerne resulted in three- and nine-fold increases in the rate of breakdown of BSA and Fraction 1 protein during incubation with rumen fluid (Nugent *et al.* 1983). Similarly, data from the present study showed that there was a higher rate of protein degradation using rumen fluid from sheep supplemented with cottonseed kernel rather than lucerne chaff.

Potential degradability of the major storage proteins (52 and 48 kDa) in cottonseed kernel was very high (average 99%), with approximately 97% of this taking

place within 8 h. Rumen degradability of dietary protein is influenced by protein content, amino acid composition and protein structure (Romagnolo *et al.* 1990). The tertiary structure of protein affects the ability of the microbial population to gain access to peptide bonds. Ovalbumin, a soluble protein with a tight, convoluted tertiary structure (Cotta and Hespell 1986), is more slowly degraded in the rumen than casein (Mangan 1972), whilst proteins with numerous cross linkages are more resistant to degradation (Nugent and Mangan 1978). Mahadevan *et al.* (1980) concluded that the level of disulphide bonding within a protein was a major factor in determining its resistance to rumen degradation. Resistant proteins, such as BSA, were rendered susceptible to degradation by treatments which destroyed disulphide bonds. As the cysteine + methionine content of 52 and 48 kDa proteins in cottonseed is much lower (1.0+0.7% and 0.7+0.5% of total amino acid residues for 52 and 48 kDa proteins, respectively; Dure and Chlan 1981) than for BSA (6.9+0.9%; Kaufmann and Hagemeister 1987) and ovalbumin (4.5+1.5%; Gilbert 1971), it seems that a low prevalence of disulphide bonds may be one of the factors contributing to the relatively high rumen degradability of globulin storage proteins in cottonseed kernel.

Although increasing rates of cottonseed hull addition reduced the potential degradability of both kernel proteins (without affecting degradation rate), the reduction was small, with equal weights of kernel and hulls (i.e. 100% hulls) reducing mean potential degradability of both kernel proteins by approximately 10% units. A similar effect was demonstrated with *in situ* potential solubility results of Yu *et al.* (1995a).

The lack of any PEG effect upon protein degradation in incubations involving cottonseed kernel only showed that PEG *per se* did not effect protein degradation. Similar observations have been reported with sheep grazing lucerne (Wang *et al.* 1995). The present study demonstrates that the application of PEG can be used to deduce effects of CT in incubations involving mixtures of cottonseed kernel and hulls, as found for forages (Barry and Forss 1983; McNabb *et al.* 1993; Wang *et al.* 1995). However, the increase in potential degradability of kernel proteins with addition of PEG accounted for only about 50% of the depression caused by the addition of hulls, suggesting that approximately half of the depression in potential degradability produced by the addition of hulls was due to CT. The remainder of the hull effect must be due to other components present in hulls although these have not been identified at this stage.

Two mg PEG mg^{-1} total CT was required to prevent or reverse the effect of CT in hulls, confirming the results obtained from total N solubility experiments (Yu *et al.* 1995a). PEG displaces CT from CT-protein complexes (Jones and Mangan 1977) and completely binds extractable CT in forage at 1.8 mg mg^{-1} total CT (Barry and Forss

1983). Thus it seems that similar concentrations of PEG are required to prevent bound CT in cottonseed binding to protein.

Whole cottonseed is a unique feedstuff with high densities of energy and protein, and has been widely used in the dairy (Coppock *et al.* 1987) and beef (Moore *et al.* 1986; Zinn and Plascencia 1992) cattle industries. Whole cottonseed supplementation often increases both milk yield and milk fat content, but decreases milk protein percentage (Coppock and Wilks 1991; Stern & Ziemer 1993). Whole cottonseed contains 33% hulls (Yu *et al.* 1993), and based upon the results of Experiment 3, this would have an almost negligible effect upon reducing the rumen degradation of kernel proteins.

Yu *et al.* (1995b) determined the effect of CT in cottonseed hulls upon ileal digestibility of amino acids in growing rats, using diets where cottonseed kernel was the sole source of protein. The overall results from rat trials showed that adding hulls (4.6% of total diet) depressed apparent ileal digestibility of amino acids from 83% to 75%, whilst inclusion of PEG in the diets containing hulls increased apparent ileal amino acid digestibility to 79%. These effects occurred with the addition of low dietary concentrations of hulls in monogastric diets and suggests that cottonseed hulls depressed digestion of cottonseed protein in the small intestine to a much greater extent than in the rumen. Assuming the same effect occurs in the small intestine of the ruminant, bound CT in cottonseed products (whole cottonseed and cottonseed meal) will probably have a minor effect in reducing dietary protein degradation in the rumen, but may have a significant effect in reducing amino acid absorption from the small intestine. Thus, bound CT in cottonseed products, unlike extractable CT in forages, may have a greater adverse than beneficial effect upon dietary protein utilisation. The difference may be that extractable CT can readily react with dietary protein during chewing by ruminant animals, whereas bound CT probably has limited capacity to move from hulls to other particles (such as kernels) at rumen pH. As some CT-protein complexes are unstable at abomasal pH values (2.0-3.0; Jones and Mangan 1977), this may release considerable amounts of cottonseed CT from binding to hulls, and account for its greater effect in depressing protein digestion in the small intestine. This needs to be investigated in future studies.

The present study has shown that incubation with rumen fluid, followed by separation of individual proteins using SDS-PAGE and quantification by densitometry was used successfully to estimate the effect of adding cottonseed hulls upon the *in vitro* degradation of storage proteins in unheated solvent extracted cottonseed kernel. Further work using the same procedures is needed to study effect of other compounds in cottonseed upon protein degradation. The present studies have been done with

unheated solvent extracted cottonseed kernel. Heating, as employed during commercial CSM manufacturing, may have some effect upon the protein degradation, so the degree of heat treatment and interactions between heat, hulls and gossypol upon the degradation of the 52 and 48 kDa cottonseed kernel proteins by rumen micro-organisms should be investigated.

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Chapter 5

EFFECT OF CONDENSED TANNIN IN COTTONSEED HULLS ON ENDOGENOUS ILEAL AMINO ACID LOSS IN THE GROWING RAT

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5.1 ABSTRACT

The effect of condensed tannin (CT) in cottonseed hulls (CSH) on endogenous ileal amino acid flow in the growing rat was evaluated. CSH contain around 900 g kg^{-1} fibre and 52 g kg^{-1} total CT. Twenty-four rats were allocated to four semi-synthetic diets, which contained enzymically hydrolysed casein (EHC) as the sole source of dietary nitrogen and chromic oxide as an indigestible marker. Two of the diets contained no CSH while the remaining two contained 50 g kg^{-1} CSH. At each level of hull inclusion (0 and 50 g kg^{-1}), polyethylene glycol (PEG; MW 3,500) was added (6 g kg^{-1}) to one of the diets. The effect of CT was assessed by determining endogenous ileal amino acid loss in the presence or absence of PEG, which binds and inactivates CT. The rats were given their respective experimental diets *ad libitum* for 14 days. Samples of digesta were collected at slaughter from the terminal 15 cm of ileum. The digesta samples were centrifuged and the supernate ultrafiltered. The precipitate plus retentate (MW> 10,000) fraction affords an estimate of endogenous loss. Inclusion of CSH in the EHC based diet increased ileal flow of total nitrogen (1387 vs 1623 mg kg^{-1} dry matter intake; $p<0.05$), increased ileal flow of total amino acids (23%; $p<0.01$), and significantly increased ileal flow of several individual amino acids. There was no significant effect of PEG and no PEG × diet interaction, showing that the CSH effects could not be explained by action of CT. The presence of hulls in commercial cottonseed meal would appear to contribute to the reported low apparent ileal amino acid digestibility coefficients for cottonseed meal by increasing endogenous ileal amino acid flow, but this effect is not due to the CT component of the hulls.

5. 2 INTRODUCTION

Commercially produced cottonseed meal (CSM), an important source of protein for monogastric farm animals, often contains significant amounts of cottonseed hulls (CSH; 15-30%; Yu *et al.* 1993). CSH contain around 900 g kg^{-1} fibre, 52 g kg^{-1} total condensed tannin (CT) and small amounts of protein (Yu *et al.* 1995b). Condensed tannins are present in the cottonseed hulls, but not in the kernel, with 80% being bound to protein and fibre (Yu *et al.* 1993). CT, recognised as being an anti-nutritional factor (ANF) in the diets of monogastric animals (Huisman 1989) may contribute to the lowered protein nutritional value of CSM (Batterham *et al.* 1990). CT are polyphenolic compounds with the ability to precipitate proteins from aqueous solutions, and are reported to increase the faecal excretion of nutrients in monogastrics, particularly amino acids (Mangan 1988). CT may also inhibit the activity of digestive enzymes due to the formation of tannin-enzyme complexes which are biologically inactive (Longstaff and McNab 1991).

In the present study the effect of CT in cottonseed hulls on endogenous ileal amino acid flow in the growing rat was determined. The recently developed peptide alimentation/digesta ultrafiltration method (Moughan *et al.* 1990; Butts *et al.* 1991) was used to determine the endogenous ileal amino acid flows. The effect of CT was assessed by determining endogenous ileal amino acid excretion in the presence or absence of polyethylene glycol (PEG; molecular weight (MW) 3,500). PEG binds strongly to CT and can be used to completely displace protein from CT-protein complexes (Jones and Mangan 1977). The effects of CT can be quantified by comparing rats receiving a control diet (CT acting) with animals given the same diet but with PEG added (CT inactivated). In the present work PEG was added at a ratio of 2 mg mg^{-1} total CT to maximise displacement of protein from the CT-protein complexes (Yu *et al.* 1995a). Work was also conducted to demonstrate that addition of PEG to the diet had no intrinsic effect on endogenous ileal amino acid flows in the absence of CT.

5. 3 MATERIALS AND METHODS

5. 3. 1 Animal and Diets

Twenty-four 180(±4.8) g, (mean±SE) Sprague-Dawley female rats were kept individually in raised stainless steel cages with wire mesh floors at 20±2°C, and with a 12 h light/dark cycle. The rats were initially fed a casein-based diet for 2 days and were then allocated at random to four experimental diets for a 14 day period.

The four semi-synthetic diets were based on maize starch, and contained enzymically hydrolysed casein (EHC; Casein peptone, New Zealand Pharmaceuticals Ltd, Palmerston North, NZ; 96% DM, free amino acids and peptides with MW<2,000 Da) as the sole nitrogen source, and chromic oxide as an indigestible marker compound (Table 5. 1). The rats were trained to consume their experimental diets between 0800 and 1800 h, with feeders being placed in the cage for 10 min at hourly intervals. The frequent feeding regimen was important to give a relatively constant flow of digesta at the terminal ileum. Fresh water was available at all times.

Table 5. 1 Ingredient composition and the condensed tannin content (g kg^{-1} air dry weight) of the enzymically hydrolysed casein (EHC) based diets

	Diet			
Cottonseed hulls	0 g kg^{-1}		50 g kg^{-1}	
PEG[1]	-	+	-	+
Enzymically hydrolysed casein[2]	100	100	100	100
Cottonseed hulls			50	50
PEG[1]		6		6
Maize starch	629	623	579	573
Sucrose	100	100	100	100
Maize oil	65	65	65	65
Purified cellulose[3]	50	50	50	50
Mineral/vitamin premix[4]	15	15	15	15
Sodium choride	5	5	5	5
Magnesium sulphate	2	2	2	2
Potassium carbonate	4	4	4	4
Dicalcium phosphate	24	24	24	24
Chromic oxide	6	6	6	6
Condensed tannin:[5]				
Total	0	0	2.6	2.6
Free	0	0	0.65	0.65

[1] Polyethylene glycol, MW 3,500.

[2] Casein peptone; New Zealand Pharmaceuticals Ltd, Palmerston North, NZ; 96% DM, free amino acids and peptides with MW<2,000 Da.

[3] Avicel, Asahi Chemical Industry Company Ltd, Tokyo, Japan.

[4] Rat Pellet Premix 9327, Technik Products, Auckland, New Zealand. Supplied the following per kg diet: 10,000 IU Vitamin A; 1,500 IU Vitamin D3; 30 IU Vitamin E; 1 mg Vitamin K; 1 mg Thiamine (B1); 4 mg Riboflavin (B2); 3 mg Pyridoxine (B6); 0.02 mg Vitamin B12; 15 mg Pantothenic acid; 1 mg Folic acid; 25 mg Niacin; 125 mg Antioxidant; 250 mg Choline; 100 mg Manganese; 35 mg Iron; 10 mg Copper; 60 mg Zinc; 1 mg Cobalt; 0.15 mg Selenium.

[5] Mean of duplicate determinations.

5. 3. 2 Sampling and Chemical Analysis

On day 14, the rats were asphyxiated in carbon dioxide gas and decapitated (immediately ceasing all neural stimulation to the gut) at 8 hours from the start of feeding, as described by Yu *et al.* (1995b). Ileal digesta were collected from the terminal 15 cm of ileum. The ileal digesta samples were centrifuged at 1,400 *g* for 45 min at 0°C, and then the supernatants were subjected to ultrafiltration using Centriprep-10 concentrators (MW exclusion limit 10,000 Daltons (Da); Amicon, W R Grace and Co., Danvers, USA). This ultrafiltration process provides molecular separation of proteins (MW>10,000 Da) from peptides and free amino acids (MW<10,000 Da). The high molecular weight fraction (retentate; MW>10,000 Da) was added back to the precipitate. The total precipitate plus retentate and diet samples were subsequently freeze-dried, finely ground and stored at -20°C for the determination of nitrogen, chromium, and amino acid concentrations.

The diets and ileal digesta were analysed in duplicate for total nitrogen using the Kjeldahl procedure. The chromium contents of duplicate 15 mg samples of ileal digesta and each diet were determined by the method of Costigan and Ellis (1987). The extractable (ie. free) and bound CT contents of the diets were determined using the method of Terrill *et al.* (1992), and the amino acid compositions of duplicate 5-7 mg samples were determined using high performance liquid chromatography (HPLC, Waters Associates, USA) as described by Yu *et al.* (1995b). Free amino acid molecular weights were used to calculate the weights of amino acids.

5. 3. 3 Calculation and Statistical Analysis

Endogenous flows of amino acids at the terminal ileum relating to the ingestion of 1 g of freeze dry matter (FDM) were calculated using the following equation:

$$\text{Amino acid (AA) flow}^{*} = \text{AA concentration in ileal digesta} \times \frac{\text{diet chromium concentration}}{\text{ileal chromium concentration}}$$

* All units were mg g-1 FDM.

The endogenous ileal amino acid flows were determined based on the amino acid contents of the precipitate plus high molecular weight fraction (MW>10,000 Da) following centrifugation and ultrafiltration. A linear statistical model, which included terms for hulls, PEG and hulls × PEG, was fitted to the data for each amino acid singly, and reduction in sums of squares was used to determine levels of significance (SAS 1985).

5. 4 RESULTS

The rats consumed the experimental diets readily and remained healthy throughout the study. The overall mean (±SE) daily food intake (days 10-13) was 11(±0.4) g. Faeces were not detected in the gastric contents at slaughter indicating that coprophagy had not occurred at least on the last day of study. Adding 50g kg^{-1} hulls to the EHC based diet led to an increase in the determined endogenous ileal flow of total nitrogen (N; $p<0.05$), total essential amino acids ($p<0.01$) and total non-essential amino acids ($p<0.01$), and of the individual amino acids leucine, serine ($p<0.01$), arginine, methionine, phenylalanine and glutamic acid ($p<0.05$; Table 5. 2). The endogenous ileal flows of other individual amino acids tended to increase with the addition of hulls, but the differences were not statistically significant ($p>0.05$). For three of the individual amino acids (threonine, proline and aspartic acid) the differences approached statistical significance ($p<0.10$). The mean increase in the estimate of the endogenous ileal loss of total amino acids, due to the addition of 50g kg^{-1} of hulls to the EHC diet, was 23% ($p<0.01$). The addition of PEG (6 g kg^{-1} DM) did not affect endogenous N or amino acid flow at the terminal ileum, in either the presence or absence of hulls in the EHC based diet. The hulls × PEG interaction was not significant ($p>0.05$).

5. 5 DISCUSSION

The peptide alimentation/digesta ultrafiltration method allowed investigation of the effect of CSH and CT on endogenous ileal amino acid flow in the growing rat. In this method the animal is fed a semi-synthetic diet containing EHC as its sole nitrogen source. Ileal digesta are collected and the nitrogenous fraction separated physically using large volume disposable ultrafiltration devices. The high molecular weight (MW>10,000 Da) fraction resulting from the ultrafiltration provides a measure of endogenous amino acid flow. If some of the dietary amino acids and small peptides are not absorbed, they will be removed in the low molecular weight fraction (MW<10,000 Da). In addition to the unabsorbed dietary amino acids and peptides, the low molecular weight fraction will contain some non-protein N, and endogenous free amino acids and small peptides. The resulting endogenous amino acid flow, therefore, will be an underestimate of the actual flow. Butts *et al.* (1991) reported that the amount of endogenous free amino acids plus small peptides in the terminal ileal digesta of the rat given an EHC based diet was low.

Table 5. 2 Mean (n=6) endogenous nitrogen and amino acid flows (mg kg^{-1} freeze dry matter intake) at the terminal ileum of the growing rat given an enzymically hydrolysed casein based diet[1] with or without cottonseed hulls and polyethylene glycol (PEG; MW 3,500)

Hulls	0 g kg^{-1}		50 g kg^{-1}		Overall	Statistical significance[2]		
PEG	-	+	-	+	SE	Hulls	PEG	H×P[3]
Endogenous flow								
Total nitrogen	1380	1394	1612	1634	106	*	NS	NS
Essential amino acid								
Arginine	355	299	464	435	48	*	NS	NS
Cystine	208	213	221	246	25	NS	NS	NS
Histidine	237	231	270	273	29	NS	NS	NS
Isoleucine	570	562	614	685	49	NS	NS	NS
Leucine	656	588	809	774	53	**	NS	NS
Lysine	340	333	402	367	38	NS	NS	NS
Methionine	123	125	164	167	15	*	NS	NS
Phenylalanine	245	240	334	305	33	*	NS	NS
Threonine	809	862	1157	1057	120	p=0.07	NS	NS
Valine	615	593	665	698	62	NS	NS	NS
Total	4158	4046	5100	5007	262	**	NS	NS
Non-essential amino acid								
Alanine	465	481	538	547	57	NS	NS	NS
Aspartic acid	860	846	958	1110	95	p=0.10	NS	NS
Glutamic acid	1763	1778	2236	2553	179	*	NS	NS
Glycine	595	567	638	651	58	NS	NS	NS
Proline	718	736	877	839	68	p=0.09	NS	NS
Serine	1108	1120	1245	1471	83	**	NS	NS
Tyrosine	222	228	275	251	28	NS	NS	NS
Total	5731	5756	6767	7422	344	**	NS	NS
Total amino acid	9889	9802	11867	12429	589	**	NS	NS

[1] Digesta were centrifuged and ultrafiltered; flows were based on amino acids in the precipitate plus retentate (M.W.>10,000).

[2] NS, no significant; *, $p<0.05$; **, $p<0.01$.

[3] H×P, hulls × PEG interaction.

The present estimates of endogenous amino acid flow for the rat given an EHC based diet (0 g kg^{-1} CSH) were slightly higher than these reported by other workers (Butts *et al.* 1991; Donkoh 1993). The inclusion of CSH in the EHC based diet increased ileal N and amino acid flows, indicating that either fibre and, or CT from CSH had an effect on the endogenous ileal loss of amino acids in the rat. However, the protein in the CSH (35 g kg^{-1}; Yu *et al.* 1995b) could have caused part of the increase in

the estimate of endogenous ileal amino acid loss. If it is assumed that the CSH protein was completely indigestible, then it can be shown that the CSH protein could possibly have contributed a 10% increase in the ileal excretion of N. Ileal N excretion increased some 23% with CSH addition, so it appears that there was a small effect of the CSH on endogenous protein loss. Given that there was no response to the dietary addition of PEG, CT in the cottonseed hulls did not appear to influence endogenous protein or amino acid flow at the terminal ileum of the rat.

Jansman (1993) fed pigs diets containing faba bean hulls with low and high concentrations of CT, and found that either dietary fibre and, or CT in faba bean led to a decreased true digestibility of dietary protein and increased excretion of endogenously secreted proteins. Shahkhalili *et al.* (1990) suggested that diets rich in polyphenols from varying sources influence faecal N excretion in the rat. The rats in the present study initially lost body weight, but after a week had started to gain in weight (data not included). A similar observation was made by Glick and Joslyn (1970) who fed rats different types of tannin, including tannic acid, and by Mehansho *et al.* (1983) who gave rats a high-tannin sorghum. The consumption of diets containing tannin was shown to specifically increase the size of the parotid glands in the rat and the synthesis and secretion of proline-rich proteins (PRPs; Mehansho *et al.* 1992). Tannin-induced PRPs were shown to have a very high binding affinity for tannins (Mehansho *et al.* 1983). The binding of tannins to both dietary and endogenous proteins has been used to explain the reduced apparent digestibility of protein in tannin containing diets. However, evidence for dietary CT increasing endogenous ileal amino acid loss was not found in the present study.

Overall, inclusion of CSH in the EHC based diet led to an increase in the ileal excretion of amino acids. There may have been some effect of the CSH fibre component on endogenous ileal amino acid loss, but the cottonseed CT did not appear to influence this loss. Based on the present result, the presence of some hulls in commercial cottonseed meal will partly contribute to the reported low apparent ileal amino acid digestibility coefficients for CSM protein. However, this cannot be explained by an effect of the CT content of the hulls on endogenous ileal amino acid loss.

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Chapter 6

THE EFFECT OF COTTONSEED CONDENSED TANNINS ON THE ILEAL DIGESTIBILITY OF AMINO ACIDS IN CASEIN AND COTTONSEED KERNEL

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6. 1 ABSTRACT

The effect of adding cottonseed hulls to casein and cottonseed kernel based diets on the apparent and true ileal digestibility of nitrogen (N) and amino acids, and the proportion of this effect accounted for by condensed tannin (CT), was determined using the growing rat. Sixty rats were allocated randomly to ten semi-synthetic diets, containing either casein (4 diets) or purified unheated solvent-extracted cottonseed kernel (6 diets) as the sole protein sources, with chromic oxide added as an indigestible marker. Two of the casein diets contained no hulls whilst the remaining two diets contained 70 g cottonseed hulls kg^{-1}. Two of the cottonseed kernel based diets contained no hulls, with two containing 23 g hulls kg^{-1} and the remaining two containing 46 g hulls kg^{-1}. For each pair of diets, polyethylene glycol (PEG) was either included or excluded. The effect of CT was quantified by comparing control rats (-PEG; CT acting) with PEG supplemented rats (+PEG; CT inactivated) at each level of dietary hulls. The rats were given their respective experimental diet for 14 days. Each rat was given the food *ad libitum* for 10 min, hourly from 0800 to 1800 h. On day 14, samples of digesta were collected at death from the terminal 15 cm of ileum at 7 hours from the first meal. Apparent and true ileal digestibilities were calculated for dry matter (DM), N and the individual amino acids. The principal finding was that the inclusion of hulls depressed the apparent and true ileal digestibility of N and amino acids, but with the response differing between diets. With the casein based diet the mean apparent and true ileal amino acid digestibilities were significantly depressed from 0.89 and 0.96 to 0.85 and 0.92, respectively, by the inclusion of 70 g hulls kg^{-1} in the diet, and addition of PEG then restored these to 0.89 and 0.95. All of the depression could be explained by the CT content of the hulls. However, with the cottonseed kernel based diet the response fell into three categories. The apparent and true ileal digestibilities of the essential amino acids cystine and methionine were not affected by hull addition, ileal digestibilities of leucine, isoleucine, lysine, threonine and valine were markedly depressed by hull addition with approximately 50% of the depression being explained by CT, whilst the ileal digestibilities of histidine, arginine and phenylalanine were depressed by hull addition but little or none of this effect could be explained by CT. Thus the effect of hulls on protein digestion clearly differed with source of protein. With the cottonseed kernel based diet it seems that components of the hulls other than CT also depressed the apparent and true ileal digestibility of N and amino acids. The identity of these components is unknown.

6. 2 INTRODUCTION

Cottonseed meal (CSM) is an important source of protein for monogastric farm animals (Lusas and Jividen 1987), but is regarded as being of variable quality and generally of low amino acid availability (Batterham *et al.* 1990; Batterham 1992). The nutritional value of CSM is influenced by the processing conditions applied to cottonseed during oil extraction, and by the presence of anti-nutritional factors (ANFs) and non-starch polysaccharides (Frank 1987; Huisman *et al.* 1990; Yu *et al.* 1993). Gossypol, a naturally-occurring polyphenolic substance in cottonseed, reacts with the *epsilon*-amino group of lysine during heating of the seed to form insoluble, indigestible complexes (Lyman *et al.* 1959; Damaty and Hudson 1979; Berardi and Goldblatt 1980; Ikurior and Fetuga 1988). Other components of the seed, such as asparagine and glutamine (Varnish and Carpenter 1975), raffinose and phospholipids (Martinez *et al.* 1967) have been found to interact during seed processing, leading to inter- and intra-molecular cross-linkages, which reduce protein digestibility by obstructing enzymic attachment (Varnish and Carpenter 1975).

Recent studies have found that condensed tannins (CT) are present in commercially produced CSM in significant concentrations (8 to 16 g kg^{-1} DM; Balogun *et al.* 1990; Terrill *et al.* 1992; Yu *et al.* 1993). CT occur in cottonseed hulls (32-65 g kg^{-1} DM) mainly bound to protein and fibre, but are absent from cottonseed kernel (Yu *et al.* 1993). Condensed tannins are polyphenolic compounds, capable of precipitating proteins from aqueous solutions, and have been shown to have anti-nutritional effects in non-ruminant animals (Huisman *et al.* 1990; Helsper *et al.* 1993). Specifically, they are known to increase faecal excretion of nutrients, particularly amino acids, thus reducing apparent nutrient digestibility (Mangan 1988; Salunkhe *et al.* 1990). Moreover, *in vitro* and *in vivo* studies have demonstrated that CT can inhibit the activity of digestive enzymes (Longstaff and McNab 1991; Jansman 1993) due to the formation of tannin-enzyme complexes which are biologically inactive (Griffiths 1979; Griffiths and Moseley 1980). In the rat, sorghum and faba bean CT are known to cause hypertrophy of the parotid glands, accompanied by an increased secretion of proline-rich proteins (Mehansho *et al.* 1983, 1992; Jansman 1993). CT may cause damage to the gut mucosa (Mitjavila *et al.* 1977; van Leeuwen *et al.* 1993), and may also cause an increased loss of mucin in the faeces (Sell *et al.* 1985).

There is no information on the effects of bound CT in cottonseed hulls on nutrient digestibility in monogastric animals. The objective of the present study was to determine the effect of CT in cottonseed hulls on the apparent and true ileal digestibility of amino acids in casein and in unheated solvent-extracted cottonseed kernel fed to the growing rat. Dietary polyethylene glycol (PEG) addition was used in the present work,

to allow an effect of the CT, consequent upon an increase in the level of dietary inclusion of cottonseed hulls, to be distinguished from an effect of the increased fibre. PEG binds strongly to CT and can be used to completely displace protein from the CT-protein complexes (Jones and Mangan 1977; Barry and Manley 1986). Work was also conducted to demonstrate that adding PEG to the diet had no effect on protein digestion in the absence of CT.

6. 3 MATERIALS AND METHODS

6. 3. 1 Preparation of Cottonseed Kernel and Hulls

Delinted whole cottonseed (var. Siokra L22) supplied by Cotton Seed Distributors Ltd, Wee Waa, NSW, Australia was cracked using a Crushing-Mill (AB Thorell and Persson, Uppsala, Sweden), and separated into kernels and hulls using air-flow, at the Seed Technology Centre, Massey University, with final manual separation. The separated kernels were freeze-dried for 48 h, ground to pass a 2 mm diameter sieve, and the oil and gossypol were extracted using hexane and then acetone in water (70:30 w/w) using a modification of the Pons and Eaves (1967) procedure, as described by Yu *et al.* (1995a). Finally, extracted cottonseed kernel and hulls were then re-ground to pass through a 1 mm diameter sieve and were stored at -20°C. The chemical composition of the unheated solvent-extracted cottonseed kernel and hulls are shown in Table 6. 1.

6. 3. 2 Animals and Diets

Male and female Sprague-Dawley rats, which had been weaned at 4 weeks of age, were reared on a high quality diet at the Small Animal Production Unit, Massey University. The animals were kept individually in raised stainless steel cages with wire mesh floors, at 20±2°C and with a 12 h light/dark cycle.

Ten semi-synthetic diets were formulated based on corn starch, and containing either casein or purified unheated solvent-extracted cottonseed kernel as the sole protein source (Table 6. 2). The diets contained graded levels of cottonseed hulls, and chromic oxide was added, as an indigestible marker compound, to all diets. At each level of dietary hulls, PEG (molecular weight (MW) 3500, Union Carbide, Danbury, CT, USA) was either included or excluded. Thus, the effect of CT can be quantified by comparing control rats (-PEG; CT acting) with PEG-supplemented rats (CT inactivated) at each level of dietary hulls. The PEG was added at a minimum ratio of 2 mg mg^{-1} total CT to maximise the displacement of protein from the CT-protein complexes (Yu *et al.* 1995b).

Table 6. 1 Chemical compositions[1] (g kg^{-1} DM) of the unheated solvent-extracted cottonseed kernel and hulls

	Cottonseed kernel	Cottonseed hulls
Dry matter (g kg^{-1})	909	911
Crude protein	537	35
Oil	138	10
Neutral detergent fibre	89	886
Acid detergent fibre	37	624
Lignin	27	209
Free gossypol	0.8	0.2
Condensed tannin:		
Extractable	0	13
Protein-bound	0	29
Fibre-bound	0	10
Total (calculated)	0	52
Essential amino acid		
Arginine	64	1.0
Histidine	16	0.5
Isoleucine	22	0.9
Leucine	43	1.4
Lysine	30	1.1
Phenylalanine	39	0.9
Threonine	21	0.9
Valine	30	1.1
Total (calculated)	265	7.8
Non-essential amino acid		
Alanine	26	1.3
Aspartic acid	7	6.9
Glutamic acid	101	2.9
Glycine	24	1.0
Proline	23	1.2
Serine	24	1.6
Tyrosine	19	1.1
Total (calculated)	224	16.0

[1] Mean of duplicate determinations.

Table 6. 2 Ingredient (g kg^{-1} air dry weight) and chemical compositions of the casein and unheated solvent-extracted cottonseed kernel based diets

	Diet									
	Casein				Cottonseed kernel					
Cottonseed hulls (g kg^{-1})	0		70		0		23		46	
PEG[1]	-	+	-	+	-	+	-	+	-	+
Ingredient										
Casein	160	160	160	160						
Cottonseed kernel					290	290	290	290	290	290
Cottonseed hulls			70	70			23	23	46	46
PEG[1]		8		8		5		2.5		5
Maize starch	599	591	529	521	469	464	446	443.5	423	418
Sucrose	100	100	100	100	100	100	100	100	100	100
Maize oil	50	50	50	50	50	50	50	50	50	50
Cellulose[2]	35	35	35	35	35	35	35	35	35	35
Mineral/vitamin premix[3]	15	15	15	15	15	15	15	15	15	15
Sodium chloride	5	5	5	5	5	5	5	5	5	5
Magnesium sulphate	2	2	2	2	2	2	2	2	2	2
Potassium carbonate	4	4	4	4	4	4	4	4	4	4
Dicalcium phosphate	24	24	24	24	24	24	24	24	24	24
Chromic oxide	6	6	6	6	6	6	6	6	6	6
Nutrient content (DM basis)[4]										
DM[5]	983	982	977	984	973	971	971	975	966	975
OM[5]	951	945	950	949	924	927	926	922	927	931
Crude protein	150	156	162	156	160	160	163	161	169	165
Oil	17	20	20	15	94	94	91	94	93	91
NDF[5]			61	61	26	26	46	46	67	67
ADF[5]			43	43	11	11	25	25	39	39
Lignin			14	14	8	8	13	13	18	18
Gross Energy (MJ kg^{-1})	18	18	18	18	18	19	19	19	19	19
Free gossypol (mg kg^{-1})					174	182	175	189	184	186
Condensed tannin (g kg^{-1})										
Total	0	0	3.6	3.6	0	0	1.2	1.2	2.4	2.4
Free	0	0	0.91	0.91	0	0	0.30	0.30	0.60	0.60

[1] Polyethylene glycol, MW 3,500.

[2] Avicel, Asahi Chemical Industry Company Ltd, Tokyo, Japan.

[3] Rat Pellet Premix 9327, Technik Products, Auckland, New Zealand. Supplied the following per kg diet: 10,000 IU Vitamin A; 1,500 IU Vitamin D3; 30 IU Vitamin E; 1 mg Vitamin K; 1 mg Thiamine (B1); 4 mg Riboflavin (B2); 3 mg Pyridoxine (B6); 0.02 mg Vitamin B12; 15 mg Pantothenic acid; 1 mg Folic acid; 25 mg Niacin; 125 mg Antioxidant; 250 mg Choline; 100 mg Manganese; 35 mg Iron; 10 mg Copper; 60 mg Zinc; 1 mg Cobalt; 0.15 mg Selenium.

[4] Means of duplicate determinations.

[5] DM, dry matter; OM, organic matter; NDF, neutral detergent fibre; ADF, acid detergent fibre.

6. 3. 3 Experimental Procedure

Sixty rats (mean±SE bodyweight, 176±4.5 g) were assigned randomly to the ten experimental diets (Table 2), such that there were three males and three females per diet. The animals were initially fed a casein-based diet (approximately 100 g crude protein kg^{-1}) for 2 days and were then given the experimental diets for a further 14 days. The diets were offered in stainless steel feeders fitted with anti-spill devices similar to those described by Thomsen (1981). The rats were trained to consume their experimental diet between 0800 and 1800 h, with the feeder being placed in the cage for 10 min at hourly intervals. The training was achieved within 7 days, and feed intakes were recorded after each 10 min feeding. Fresh water was freely available.

On day 14, the rats were asphyxiated in carbon dioxide gas and decapitated (immediately ceasing all neural stimulation to the gut) at 7 hours from the start of feeding. The abdomen was opened by an incision along the mid-ventral line and the skin and musculature were folded back to expose the viscera. The final 15 cm of the ileum was immediately dissected from the body, and the intestinal surface cleaned using absorbent tissue paper, taking care not to apply pressure to the intestine. The digesta were slowly flushed out into plastic bags with distilled water from a plastic syringe. The digesta from each animal were kept separate and packed in ice immediately after collection.

Ileal digesta and samples of the test diets were subsequently freeze-dried, finely ground and stored at -20°C for the determination of nitrogen, chromium, and total amino acids. The stomach contents were inspected for signs of faecal contamination resulting from coprophagy.

6. 3. 4 Chemical Analysis

The diets and ileal digesta were analysed in duplicate for total nitrogen using the Kjeldahl procedure, and crude protein calculated as total N × 6.25. The chromium contents of duplicate 15 mg samples of ileal digesta and each diet were determined by the method of Costigan and Ellis (1987). The CT contents of the diets were determined using the method of Terrill *et al.* (1992). Free gossypol in the diets was estimated by the method Ba 7-58 of AOCS (1975). The NDF, ADF and lignin contents were determined by the method of Robertson and van Soest (1981). The crude ash, crude oil and gross energy contents of the feeds were analysed according to conventional methods (AOAC 1975). The amount of freeze-dried matter (FDM) collected from the terminal ileum of each rat was determined after freeze drying the samples for 3 days.

Amino acid composition was determined on 5-7 mg samples using high performance liquid chromatography (HPLC, Waters Associates, USA), using a reverse phase column and the Pico.Tag analytical method (Cohen *et al.* 1989). Duplicate samples were hydrolysed in 500 µl of 6 M HCl with 1% added phenol, for 24 hours at 110±1°C in glass tubes sealed under vacuum. For the determination of methionine and cystine in the samples obtained from the cottonseed kernel based diets, separate duplicate samples were oxidised with 90:10 (v/v) of performic acid:H_2O_2 prior to hydrolysis. Methionine and cystine in the samples obtained from the casein based diet, and tryptophan, which was partly destroyed during acid hydrolysis, were not determined. The amino acids were detected by the fluorescence of their phenylisothiocyanate (PITC) derivatives using a programmable multi-wavelength detector (Waters 490E, USA). Free amino acid molecular weights were used to calculate the weights of amino acids.

6. 3. 5 Data Analysis

Apparent and true amino acid digestibility coefficients were calculated using the following equations:

$$\text{Apparent amino acid (AA) digestibility*} = \frac{\text{Dietary AA intake} - \text{ileal AA output}}{\text{Dietary AA intake}}$$

$$\text{True amino acid (AA) digestibility*} = \frac{\text{Dietary AA intake} - (\text{ileal AA output} - \text{endogenous AA output})}{\text{Dietary AA intake}}$$

$$\text{Amino acid (AA) output*} = \text{AA concentration in ileal digesta} \times \frac{\text{diet total chromium}}{\text{ileal total chromium}}$$

* All units were mg g^{-1} FDM.

Endogenous amino acid flows used for calculating true amino acid digestibility coefficients in the present study were obtained in a separate but related study (Yu *et al.* 1995c), in which endogenous ileal amino acid flows were determined in rats given diets containing graded levels of cottonseed hulls. The enzymically hydrolysed casein (EHC) ultrafiltration method (Moughan *et al.* 1990; Butts *et al.* 1991) was used in the latter work to determine endogenous amino acid losses. Data from the present study were subjected to ANOVA. A linear statistical model, which included terms for hulls, PEG and hulls × PEG, was initially fitted to the digestibility data for each amino acid singly, and reduction in sums of squares was used to determine levels of significance. Relevant comparisons between treatment means were made using orthogonal contrasts (Snedecor and Cochran 1982). Where a cause and effect trend in the data was expected (e.g. ileal

digestibility as a function of hull addition), the data (n=18) were subjected to a simple linear regression and slopes were tested for statistical significance from zero.

6. 4 RESULTS

The overall mean (±SE) liveweight for the rats at the end of the study was 197±8.4 g. Mean food intakes for the rats on day 13 of the study are given in Table 3. Food intake was within the normal range for the 200 g bodyweight rat (NRC 1978). Daily food intake tended to be lower with the casein based diet in comparison with the cottonseed kernel based diet, and dietary PEG addition did not affect food intake on either diet (Table 6. 3). On the last day of study, the rats had high food intakes over the first two hourly meals and then consumed generally even sized meals for the remainder of the feeding period (Table 6. 3). The latter was important to ensure an even flow of digesta at the terminal ileum. Faeces were not detected in the gastric contents at slaughter indicating that coprophagy had not occurred at least on the last day of study.

Table 6. 3 Mean food intakes[1] of the growing rats on day 13 and hourly meal intakes for the last day (day 14) of study

Diet	Food intake on day 13	Hourly meal intakes on day 14[2] (g)							
	(g)	1	2	3	4	5	6	7	Total
Casein:									
-PEG[3]	10±0.3	1.4	1.2	1.2	0.6	0.7	0.6	0.8	6.5
+PEG	9±0.7	1.4	1.0	0.9	0.6	0.6	1.0	1.0	6.5
70 g kg^{-1} hulls-PEG	13±0.7	2.7	1.9	1.7	1.0	0.8	0.9	1.2	10.2
70 g kg^{-1} hulls+PEG	13±1.5	2.1	1.9	1.6	1.1	0.8	1.2	1.3	10.0
Cottonseed kernel:									
-PEG	15±0.7	3.5	2.5	0.6	0.6	1.2	2.1	1.5	12.0
+PEG	15±0.6	3.1	2.3	1.4	1.0	0.9	1.3	1.2	11.2
23 g kg^{-1} hulls-PEG	15±1.0	3.4	2.5	1.9	1.7	0.8	1.4	1.2	12.9
23 g kg^{-1} hulls+PEG	16±0.9	3.4	2.8	1.4	1.2	0.8	1.6	1.5	12.7
46 g kg^{-1} hulls-PEG	16±0.9	3.9	2.6	2.1	0.7	1.0	1.3	1.2	12.8
46 g kg^{-1} hulls+PEG	16±1.1	3.7	3.2	2.1	0.8	0.8	1.5	1.4	13.5

[1] Each value represents the mean±SE or the mean (n=6).

[2] Day of sampling ileal digesta.

[3] Polyethylene glycol, MW 3,500.

There was a significant ($p<0.05$) hulls × PEG interaction for amino acid digestibility for several of the amino acids. Accordingly, relevant comparisons were made between treatment means within the PEG or hull factors.

With both the casein and cottonseed kernel based diets, inclusion of PEG in the diet without hulls did not affect ileal DM digestibility or the apparent and true ileal digestibility of nitrogen and individual amino acids (Tables 6. 4, 6. 5 and 6. 6), indicating that there was no effect of PEG *per se* on dietary DM and protein digestibility in the absence of CT.

With the casein based diet, in the absence of PEG, addition of dietary cottonseed hulls (70 g kg^{-1}) significantly depressed ileal DM digestibility ($p<0.001$; Table 6. 4) and the true ileal digestibility of total N ($p<0.001$). In the presence of hulls, dietary supplementation with PEG significantly increased ileal DM digestibility ($p<0.01$) and the true ileal digestibility of total N ($p<0.01$), with the values for total N attained being similar to those for diets not containing hulls.

Apparent and true ileal amino acid digestibility for rats fed the casein based diet were significantly depressed ($p<0.05$) by addition of dietary hulls (Table 6. 4). On average, the apparent ileal amino acid digestibility was decreased from 0.89 to 0.85 by the inclusion of 70 g hulls kg^{-1} in the diet, and addition of PEG then restored this to 0.89. The mean true ileal digestibility of amino acids decreased from 0.96 for the diet not containing hulls to 0.92 for the diet containing 70 g hulls kg^{-1}. When PEG was added to the diet containing hulls (70 g kg^{-1}), the mean true ileal amino acid digestibility was restored to 0.95. Apparent and true ileal digestibility of all individual amino acids were similarly affected by the addition of hulls and PEG.

With the cottonseed kernel based diet, in the absence of PEG, inclusion of dietary hulls progressively depressed ($p<0.05$) ileal DM digestibility and apparent ileal digestibility of total N for the rats (Table 6. 5) in a linear manner ($p<0.05$; Table 6. 7). Addition of dietary PEG significantly increased the apparent ileal N digestibility ($p<0.05$), but did not affect ileal DM digestibility. However, the increased values for total N digestibility accounted for only about 67 and 50% of the depression in the ileal digestibility caused by the inclusion of different levels of dietary hulls (23 and 46 g kg^{-1}), respectively. True ileal N digestibility was also linearly depressed by increased dietary hulls ($p<0.001$; Table 6. 6), and addition of PEG to the diets significantly restored this ($p<0.05$ at 46 g hulls kg^{-1} level), but not back to the original level.

In the absence of PEG, inclusion of hulls in the cottonseed kernel based diet significantly reduced ($p<0.05$) the apparent and true ileal digestibilities of all amino acids except methionine and cystine (Tables 6. 5 and 6. 6). The significant linear regression relationships (Table 6. 7) indicated that as the level of dietary hull increased, in the absence of dietary PEG, the apparent and true ileal amino acid digestibilities of all amino acids except cystine and methionine decreased in a linear manner, with the slopes of the regression lines being significantly different from zero ($p<0.05$). The coefficients of

Table 6. 4 Mean (n=6) apparent and true digestibility of dry matter, nitrogen and amino acids at the terminal ileum of the growing rat given a casein-based diet

	Apparent digestibility								True digestibility							
Hulls (g kg^{-1})[1]	0		70		Overall	Significance[3]			0		70		Overall	Significance[3]		
PEG[2]	-	+	-	+	SE	PNH	HNP	PWH	-	+	-	+	SE	PNH	HNP	PWH
Dry matter	0.84	0.84	0.69	0.77	0.017	NS	***	**								
Nitrogen	0.88	0.88	0.87	0.88	0.013	NS	NS	NS	0.94	0.94	0.90	0.93	0.004	NS	***	**
Essential amino acid																
Arginine	0.93	0.93	0.89	0.92	0.006	NS	***	**	0.98	0.98	0.95	0.97	0.004	NS	***	**
Histidine	0.93	0.93	0.87	0.92	0.006	NS	***	***	0.98	0.98	0.93	0.97	0.005	NS	***	***
Isoleucine	0.84	0.86	0.79	0.84	0.012	NS	*	*	0.93	0.93	0.88	0.91	0.008	NS	***	**
Leucine	0.94	0.95	0.91	0.94	0.004	NS	***	**	0.98	0.98	0.95	0.97	0.003	NS	***	**
Lysine	0.94	0.96	0.92	0.95	0.004	*	**	**	0.98	0.99	0.95	0.98	0.004	NS	***	***
Phenylalanine	0.95	0.96	0.93	0.96	0.004	NS	***	***	0.98	0.99	0.96	0.98	0.003	NS	***	**
Threonine	0.82	0.85	0.78	0.83	0.010	*	**	**	0.91	0.93	0.89	0.92	0.008	NS	*	*
Valine	0.87	0.89	0.84	0.88	0.008	NS	**	**	0.95	0.96	0.91	0.93	0.007	NS	**	*
Non-essential amino acid																
Alanine	0.86	0.89	0.81	0.86	0.009	**	**	**	0.95	0.96	0.90	0.94	0.007	NS	***	**
Aspartic acid	0.85	0.87	0.80	0.85	0.010	NS	**	**	0.94	0.95	0.90	0.94	0.007	NS	***	**
Glutamic acid	0.88	0.89	0.82	0.88	0.007	NS	***	***	0.94	0.95	0.90	0.93	0.008	NS	**	*
Proline	0.93	0.94	0.89	0.92	0.004	NS	***	***	0.98	0.98	0.95	0.97	0.003	NS	***	***
Serine	0.72	0.74	0.67	0.74	0.015	NS	*	*	0.88	0.89	0.82	0.86	0.010	NS	***	**
Tyrosine	0.96	0.96	0.93	0.96	0.004	NS	**	***	0.99	0.99	0.97	0.98	0.005	NS	*	NS

[1] Fractions of cottonseed hulls in the diet. [2] Polyethylene glycol, MW 3,500.

[3] PNH: PEG+ vs PEG-, 0 g kg^{-1} hulls; HNP: 0 g kg^{-1} vs 70 g kg^{-1} hulls without PEG; PWH: PEG+ vs PEG-, 70 g kg^{-1} hulls;.
NS, non significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Table 6. 5 Mean (n=6) apparent ileal dry matter, nitrogen and amino acid digestibilities for the growing rat given an extracted cottonseed kernel based diet

Hulls[1]	0 g kg^{-1}		23 g kg^{-1}		46 g kg^{-1}		Overall	Statistical significance[3]				
PEG[2]	-	+	-	+	-	+	SE	Hull effect[4]		PEG effect[5]		
								0v23	0v46	0H	23H	46H
Dry matter	0.76	0.76	0.73	0.74	0.73	0.71	0.008	*	*	NS	NS	NS
Nitrogen	0.88	0.89	0.86	0.88	0.85	0.87	0.008	NS	*	NS	*	*
Essential amino acid												
Arginine	0.94	0.94	0.91	0.92	0.90	0.91	0.007	**	***	NS	NS	NS
Cystine	0.76	0.76	0.84	0.85	0.77	0.81	0.013	***	NS	NS	NS	*
Histidine	0.88	0.88	0.82	0.83	0.79	0.80	0.012	***	***	NS	NS	NS
Isoleucine	0.81	0.80	0.78	0.80	0.61	0.75	0.018	NS	***	NS	NS	***
Leucine	0.82	0.80	0.79	0.81	0.73	0.77	0.013	NS	***	NS	NS	*
Lysine	0.82	0.80	0.77	0.78	0.71	0.74	0.015	*	***	NS	NS	NS
Methionine	0.84	0.84	0.85	0.87	0.84	0.86	0.013	NS	NS	NS	NS	NS
Phenylalanine	0.88	0.86	0.86	0.87	0.82	0.84	0.011	NS	**	NS	NS	NS
Threonine	0.74	0.73	0.64	0.70	0.63	0.67	0.015	***	***	NS	**	*
Valine	0.83	0.82	0.79	0.81	0.72	0.78	0.014	*	***	NS	NS	**
Non-essential amino acid												
Alanine	0.79	0.78	0.75	0.78	0.73	0.77	0.014	NS	**	NS	NS	*
Aspartic acid	0.83	0.83	0.77	0.81	0.76	0.78	0.012	**	***	NS	*	NS
Glutamic acid	0.90	0.90	0.88	0.89	0.84	0.87	0.007	*	***	NS	NS	*
Glycine	0.78	0.76	0.73	0.73	0.62	0.75	0.018	*	***	NS	NS	***
Proline	0.82	0.81	0.75	0.79	0.70	0.73	0.010	***	***	NS	*	*
Serine	0.83	0.82	0.75	0.78	0.71	0.75	0.016	**	***	NS	NS	*
Tyrosine	0.88	0.89	0.84	0.86	0.80	0.82	0.014	*	***	NS	NS	NS

[1] Fractions of cottonseed hulls in the diet. [2] Polyethylene glycol, MW 3,500. [3] NS, non significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

[4] Effect of hulls in diets without PEG. 0v23: 0 g kg^{-1} hulls vs 23 g kg^{-1} hulls; 0v46: 0 g kg^{-1} hulls vs 46 g kg^{-1} hulls.

[5] Effect of PEG within level of hull inclusion. 0H: 0 g kg^{-1} hulls; 23H: 23 g kg^{-1} hulls; 46H: 46 g kg^{-1} hulls.

Table 6. 6 Mean (n=6) true ileal nitrogen and amino acid digestibilities for the growing rat given an extracted cottonseed kernel based diet

Hulls[1]	0 g kg^{-1}		23 g kg^{-1}		46 g kg^{-1}		Overall	Statistical significance[3]				
PEG[2]	-	+	-	+	-	+	SE	Hull effect[4]		PEG effect[5]		
								0v23	0v46	0H	23H	46H
Nitrogen	0.93	0.94	0.90	0.91	0.88	0.90	0.005	***	***	NS	NS	*
Essential amino acid												
Arginine	0.95	0.96	0.94	0.94	0.93	0.94	0.007	NS	*	NS	NS	NS
Cystine	0.85	0.86	0.88	0.90	0.86	0.88	0.011	*	NS	NS	NS	*
Histidine	0.93	0.94	0.90	0.91	0.87	0.88	0.012	NS	***	NS	NS	NS
Isoleucine	0.94	0.94	0.92	0.93	0.88	0.91	0.015	NS	**	NS	NS	*
Leucine	0.88	0.88	0.86	0.87	0.82	0.85	0.010	NS	***	NS	NS	*
Lysine	0.86	0.86	0.83	0.84	0.76	0.81	0.011	*	***	NS	NS	**
Methionine	0.89	0.90	0.89	0.91	0.90	0.91	0.010	NS	NS	NS	NS	NS
Phenylalanine	0.90	0.90	0.88	0.90	0.86	0.87	0.009	NS	**	NS	NS	NS
Threonine	0.87	0.87	0.80	0.85	0.79	0.82	0.011	***	***	NS	**	*
Valine	0.92	0.93	0.90	0.92	0.87	0.89	0.011	NS	**	NS	NS	*
Non-essential amino acid												
Alanine	0.87	0.87	0.82	0.85	0.79	0.83	0.012	*	***	NS	NS	*
Aspartic acid	0.91	0.92	0.87	0.90	0.86	0.87	0.013	*	*	NS	NS	NS
Glutamic acid	0.97	0.98	0.97	0.98	0.95	0.95	0.006	NS	**	NS	NS	NS
Glycine	0.86	0.85	0.78	0.82	0.75	0.80	0.022	**	**	NS	NS	NS
Proline	0.96	0.97	0.93	0.95	0.90	0.92	0.011	NS	**	NS	NS	*
Serine	0.94	0.94	0.91	0.93	0.88	0.90	0.014	NS	*	NS	NS	NS
Tyrosine	0.94	0.95	0.91	0.92	0.88	0.90	0.012	NS	***	NS	NS	NS

[1] Fractions of cottonseed hulls in the diet. [2] Polyethylene glycol, MW 3,500. [3] NS, non significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

[4] Effect of hulls in diets without PEG. 0v23: 0 g kg^{-1} hulls vs 23 g kg^{-1} hulls; 0v46: 0 g kg^{-1} hulls vs 46 g kg^{-1} hulls.

[5] Effect of PEG within level of hull inclusion. 0H: 0 g kg^{-1} hulls; 23H: 23 g kg^{-1} hulls; 46H: 46 g kg^{-1} hulls.

determination ranged from 0.38 to 0.85, indicating that a considerable proportion of the variation in ileal digestibility was explained by fitting the regression model. Unlike the responses obtained with casein, adding hulls to a cottonseed kernel based diet depressed apparent and true ileal digestibility of some amino acids more than others, with histidine, isoleucine, leucine, lysine, threonine and valine being the essential amino acids most depressed per unit of hulls added. Glycine was the non-essential amino acid most depressed.

Table 6. 7 Linear regression relationships (n=18) between ileal dry matter, nitrogen or amino acid digestibility (Y) and hull addition (x; g kg^{-1}) for growing rats fed an unheated solvent extracted cottonseed kernel based diet not including polyethylene glycol (PEG)

	Apparent ileal digestibility			True ileal digestibility		
	Linear regression (Y)	R^2	Slope Significance[1]	Linear regression (Y)	R^2	Slope Significance[1]
Dry matter	0.756 - 0.786x	0.31	*			
Nitrogen	0.880 - 0.679x	0.41	*	0.929 - 1.025x	0.66	***
Essential amino acid						
Arginine	0.936 - 0.893x	0.63	**	0.952 - 0.468x	0.29	*
Cystine	0.789 + 0.212x	0.01	NS	0.861 + 0.200x	0.02	NS
Histidine	0.876 - 2.072x	0.77	***	0.935 - 1.469x	0.60	***
Isoleucine	0.829 - 4.301x	0.74	***	0.941 - 1.217x	0.41	**
Leucine	0.826 - 1.960x	0.58	***	0.882 - 1.286x	0.59	***
Lysine	0.817 - 2.288x	0.72	***	0.863 - 2.055x	0.69	***
Methionine	0.847 - 0.108x	0.01	NS	0.887 - 0.318x	0.06	NS
Phenylalanine	0.878 - 1.186x	0.51	**	0.903 - 0.980x	0.44	**
Threonine	0.730 - 2.630x	0.64	**	0.860 - 1.704x	0.57	***
Valine	0.838 - 2.449x	0.70	***	0.924 - 1.100x	0.41	**
Non-essential amino acid						
Alanine	0.790 - 1.319x	0.38	*	0.868 - 1.686x	0.43	**
Aspartic acid	0.822 - 1.585x	0.62	***	0.903 - 1.014x	0.33	*
Glutamic acid	0.901 - 1.297x	0.75	***	0.977 - 0.573x	0.40	*
Glycine	0.783 - 3.197x	0.74	***	0.851 - 2.360x	0.41	**
Proline	0.819 - 2.688x	0.85	***	0.954 - 1.110x	0.60	***
Serine	0.823 -2.635x	0.85	***	0.939 -1.217x	0.56	**
Tyrosine	0.876 - 1.712x	0.75	***	0.940 - 1.314x	0.52	**

[1] Significance of slope from zero; NS, non significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

In the diet with 23 g hulls kg^{-1}, supplementation with dietary PEG significantly increased the apparent and true ileal digestibility of threonine and increased the apparent ileal digestibility of aspartic acid and proline (Tables 6. 5 and 6. 6). With the rate of hull

addition increased to 46 g kg^{-1}, the apparent and true ileal digestibilities of cystine, leucine, isoleucine, threonine, valine, alanine and proline were significantly increased with PEG addition. However, unlike the casein based diet, inclusion of dietary PEG in the diets containing hulls did not restore apparent and true ileal amino acid digestibilities to the same levels as found for diets not containing hulls and not all amino acids were affected similarly. Both the apparent and true ileal digestibilities of arginine, histidine, methionine, phenylalanine, aspartic acid and tyrosine were not significantly increased by PEG addition to diets containing cottonseed hulls.

6. 5 DISCUSSION

In the present study, the effect of cottonseed hulls on the nutritional value of casein and unheated solvent extracted cottonseed kernel as dietary protein sources was studied in the growing rat by determining apparent and true ileal nitrogen and amino acid digestibility. The principal finding was that the inclusion of hulls depressed the apparent and true ileal digestibility of N and amino acids, but with the response differing between diets. With the casein based diet all amino acids were significantly affected and all of the depression in digestibility could be explained by the CT content of the hulls. However, with the cottonseed kernel based diet the response fell into three categories. The apparent and true ileal digestibility of the essential amino acids cystine and methionine were not affected by hull addition, ileal digestibilities of leucine, isoleucine, lysine, threonine and valine were markedly depressed by hull addition with approximately 50% of the depression being explained by CT, whilst ileal digestibilities of histidine, arginine and phenylalanine were depressed by hull addition but little or none of this effect could be explained by CT. Thus interactions with hulls, affecting amino acid digestion, clearly differed between sources of protein. With the cottonseed kernel based diet it seems that components of the hulls other than CT also depressed the apparent and true ileal digestibility of N and amino acids. The identity of these components is unknown.

These studies have shown the presence of hulls to be one of the reasons for the generally low levels of amino acid digestibility in cottonseed meal, due to the effect of hulls in lowering the true ileal digestibility of amino acids (present study) and in increasing endogenous ileal amino acid excretion (Yu *et al.* 1995c). The reduction in true ileal amino acid digestibility per unit increase in hull content for the cottonseed kernel diets differed for each amino acid, showing that inclusion of hulls will unbalance the digestible amino acids in CSM. This seems particularly important for amino acids that are most likely to be limiting, such as lysine and threonine, whose true ileal digestibility was most lowered by hull inclusion in the cottonseed kernel diets.

PEG has been used to absorb plant phenolics during the extraction of enzymes (Jones 1965) and preferentially binds with CT, preventing CT from reacting with proteins or carbohydrates and displaces CT from CT:protein/carbohydrate complexes (Jones and Mangan 1977; Barry and Manley 1986). If PEG is included in a CT-containing diet, the CT will still be present, but will be rendered unreactive in the digestive tract because of the preferential nature of the binding between CT and PEG (McNabb 1991). This provides a unique way of studying the effect of the presence or absence of reactive CT, without affecting the nutritive composition of the diet. Complete control of the levels of other dietary ingredients is afforded. In the present study, there was no effect of PEG addition on food intake or apparent and true ileal protein and amino acid digestibility in the diets not containing cottonseed hulls, thus demonstrating that PEG addition *per se* had no intrinsic effect on food intake and ileal protein digestibility in the absence of CT. Similar observations on endogenous ileal amino acid loss with rats fed an enzymically hydrolysed casein based diet were made by Yu *et al.* (1995c). Yu *et al.* (1995b) reported that 2 mg PEG mg^{-1} total CT was required to inactivate the CT in cottonseed hulls. It is thus assumed that in the present study PEG completely bound the CT released during digestion of cottonseed hulls and that for the hull-containing diets, comparisons, with or without PEG, represented effects of cottonseed CT.

Cottonseed hulls are a by-product of cottonseed processing, with the cottonseed being cleaned and partially dehulled before the kernels are crushed and subjected to oil extraction. Commercial cottonseed meal produced in Australia contains between 150 to 300 g hulls kg^{-1} and has a CT content of 8 to 15 g kg^{-1} DM, 92% of which is bound to protein and fibre (Yu *et al.* 1993). Extractable CT as found in fresh forages can readily react with dietary protein during ingestion and digestion (Barry and Manley 1986; McNabb 1991), but the effects of bound CT have not been previously studied in monogastric animals. Yu *et al.* (1995a) found that bound CT were relatively unreactive at rumen pH (pH 7.0), but the responses to PEG in diets containing cottonseed hulls in the present study suggest that CT were reacting with proteins in the monogastric digestive system. CT are known to be solubilised in the stomach (Jones and Mangan 1977) and the most probable explanation for the present result is that the bound CT in cottonseed hulls were solubilised and released in the stomach and were thus available to react with proteins in the small intestine.

The effects of CT in depressing apparent ileal protein digestibility may be explained either by a direct binding of CT to dietary proteins, by a reduced activity of protein-degrading enzymes (Longstaff and McNab 1991), or by increased secretion of endogenous proteins (digestive enzymes, mucus or mucosal cells; Mangan 1988; Marquardt 1989). However, in recent work where ileal endogenous amino acid flow

was determined in the rat, no effect of CT in cottonseed hulls was found (Yu *et al.* 1995c).

There were differences in the apparent ileal digestibility of amino acids between casein and cottonseed kernels. In the absence of hulls, the apparent ileal amino acid digestibility, except that for three amino acids (arginine, glutamic acid and serine), was markedly higher for the casein-based diet than for the cottonseed kernel-based diet. The difference in apparent ileal amino acid digestibility between the two protein sources suggests a difference in the intrinsic quality of the proteins. This is confirmed by the lower true ileal amino acid digestibility in the cottonseed kernel based diet than in the casein based diet (Tables 6. 4 and 6. 6). The relatively low digestibility of amino acids in cottonseed meal has been reported by other workers (Batterham *et al.* 1990; Batterham 1992). Apparent ileal amino acid digestibility was depressed by an increase in dietary hulls in both the casein and cottonseed kernel based diets. This effect may be due to both the CT and fibre contents of the hulls. However, ileal amino acid digestibility for the casein diet was still higher than that for the cottonseed kernel diet, even though the level of hull addition was higher in the casein based diet (70 g hulls kg^{-1}, compared with 23 and 46 g hulls kg^{-1} in the cottonseed based diet). With addition of PEG to the casein based diet containing hulls, the depressed ileal amino acid digestibility was restored to almost the original level, but only about half of the decrease in digestibility was restored after PEG addition with the cottonseed kernel based diet. This suggests that only about half of the depression in ileal digestibility caused by hull addition to a cottonseed kernel diet can be explained by CT, with the remainder presumably being due to unidentified reactions with other hull components.

It seems that CT may have a different affinity for proteins with different amino acid profiles. Asquith and Butler (1986) in an *in vitro* study noted that CT/protein interaction may be specific for different tannins as well as for different proteins. The high degree of interaction indicated that the differences in affinity were functionally significant. Hagerman and Butler (1981) found that sorghum tannins have a high affinity for proteins that are relatively large, with an open, loose structure and that are rich in hydrophobic amino acids, particularly proline. Cousins *et al.* (1981) in a study with sorghums containing different levels of CT showed that the apparent ileal digestibilities of tryptophan, histidine, glycine and proline were more depressed than for other amino acids in high-tannin varieties. Differences in protein structure and composition may account for the different responses to cottonseed CT observed in this study between casein and cottonseed kernel. In the present study, the true ileal digestibilities of the essential amino acids histidine, leucine, isoleucine, lysine, threonine and valine in the cottonseed kernel based diets were more depressed with high levels of dietary CT

compared with the other amino acids, suggesting that reactions between these amino acids and CT are not completely reversible in the small intestine.

The ileal digestibility coefficients presented here indicate that the correction of apparent ileal N and amino acid digestibility for endogenous excretion, as determined by the enzymically hydrolysed casein (EHC) technique (Yu *et al.* 1995c), results in true ileal N and amino acid digestibilities which are markedly higher (about 11% for alanine, cystine, aspartic acid, and up to 22% for serine) than corresponding apparent estimates. The ileal endogenous excretion of N and amino acids, determined using the recently developed peptide alimentation/digesta ultrafiltration method (Moughan *et al.* 1990; Butts *et al.* 1991), was slightly increased by the inclusion of 50 g cottonseed hulls kg^{-1} in the EHC based diet (Yu *et al.* 1995c). The authors concluded that this increase was caused by the cottonseed hull fibre component, and there did not appear to be an effect of CT on endogenous ileal amino acid loss. In the present study the lowered apparent ileal digestibility of N and amino acids in the diets containing cottonseed hulls can be attributed to a decrease in digestion and absorption as reflected by the true coefficients of digestibility of dietary protein (Tables 6. 4, 6. 5 and 6. 6) and also increased endogenous protein excretion.

The consumption of diets containing CT has been shown to increase the size of the parotid glands in the rat and the synthesis and secretion of proline-rich proteins (PRPs; Mehansho *et al.* 1992). Tannin-induced PRPs were shown to have a very high binding affinity for tannins (Mehansho *et al.* 1983). The mechanism by which tannins induce hypertrophy in the parotid glands of the rat and increase the secretion of PRPs is not clear (Jansman 1993). Although saliva of various species contain proline-rich proteins (Mole *et al.* 1990), it is not clear whether species others than the rat are able to develop a similar response when consuming tannin-containing diets. In the hamster, this response was absent and used as an explanation for the high sensitivity of this species to dietary tannin (Mehansho *et al.* 1987). If pigs and poultry lack this response, the rat may not be a completely valid model for establishing the nutritional effects of dietary tannins in these commercially important animals. Thus, further experiments studying the effects of cottonseed CT on ileal N and amino acid digestibility in pigs and poultry are necessary.

The presence of CT in the hulls is part of the host plant resistance mechanism in cotton for defence against attack by insects and pathogenic micro-organisms (Fitt *et al.* 1992). It seems, on this basis, unrealistic to select for low CT levels in cotton breeding programs. Heating, as employed during commercial CSM manufacturing, may have some effect on protein solubilization. The present study has been done with unheated solvent extracted cottonseed, so further work is needed to study the effects of hull

inclusion in the presence and absence of heat treatment, and to compare the effect of CT between animal species, notably comparing rats with pigs and poultry. If cottonseed hulls increase endogenous ileal amino acid loss (Yu *et al.* 1995c) and depress ileal protein digestion in heated extracted cottonseed kernel to the same extent as found in this study, then it seems that the presence of hulls in commercial CSM should be reduced to the lowest possible levels.

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Chapter 7

EFFECT OF HEAT TREATMENT UPON THE CHEMICAL COMPOSITION OF COTTONSEED MEAL AND UPON THE REACTIVITY OF COTTONSEED CONDENSED TANNINS

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7.1 ABSTRACT

The effects of heat treatment on the chemical composition of cottonseed meal (CSM), with or without the addition of cottonseed hulls (containing condensed tannins; CT), and upon reactivity of the CT were studied. Heat was applied in a forced draught oven at 100°C for 2 h. Fluorodinitrobenzene (FDNB) available lysine, free gossypol, extractable and bound CT concentrations, *in vitro* total nitrogen (N) solubility and the *in vitro* rumen degradation of the two major seed proteins (52 and 48 kDa) present in cottonseed kernel (which does not contain CT) were determined. The reactivity of CT was assessed by determining N solubility and rumen degradation of cottonseed kernel proteins in the presence or absence of polyethylene glycol (PEG; molecular weight (MW) 3,350), which binds and inactivates CT. Heat treatment reduced the concentrations of free gossypol and FDNB available lysine by small amounts, reduced measurable total CT content by 13%, reduced the solubility of total N, and reduced potential degradability of the 52 and 48 kDa cottonseed storage proteins by mixed rumen micro-organisms. Addition of hulls further depressed solubility of total N and ruminal degradation of the two major storage proteins in cottonseed kernel. The action of PEG *in vitro* indicated that only part of the depression caused by hull addition could be explained by the presence of CT in the hulls, and that the effects of CT upon N solubility and potential degradability in heated CSM were similar to that in unheated CSM. Addition of hulls also substantially reduced FDNB available lysine. In commercially produced materials, CSM from the Brisbane mill had a lower total CT content, lower N solubility and lower ruminal protein degradation rate than CSM from the Narrabri mill, but a similar level of FDNB available lysine. Although application of heat inactivated 13% of the total CT, such that it could no longer be extracted and detected with butanol/HCl, it did not seem to change the overall effects produced by CT in reducing N solubility and protein degradation. The effect of hull addition in reducing available lysine has considerable relevance for feeding CSM to monogastric livestock. Interactions involving heat, hulls and CT need to be further studied.

7.2 INTRODUCTION

Heat treatment is well known to affect the nutritional value of protein. Heat treatment can decrease proteolysis by blocking reactive sites for microbial proteolytic enzymes and by inactivation of enzyme inhibitors (Broderick and Craig 1980), and has been used to decrease the rumen degradation of cottonseed proteins and to increase the supply of dietary protein to the duodenum (Goetsch and Owens 1985). However, heating of cottonseed meal (CSM) during commercial processing, beyond that required to bind free gossypol to the ε-amino group of lysine and to drive off residual hexane solvent, has been shown to be detrimental to protein utilisation in monogastric animals (Tanksley and Knabe 1981; Batterham 1992). This reduction in quality appears to be due partly to reduced protein digestibility and partly to reduced lysine availability (Hurrell and Carpenter 1977; Batterham *et al.* 1990). The loss of available lysine in CSM is also partly due to its reaction with gossypol during processing (Frank 1987).

Previous studies have established the presence of condensed tannin (CT) in cottonseed hulls, but not in kernels (Yu *et al.* 1993), and have shown that CT depressed hull fibre digestion in the rumen (Yu *et al.* 1995a). The solubility and degradability of protein in unheated solvent extracted cottonseed kernels in the rumen was reduced by the addition of hulls, with approximately 50% of the depression in degradability caused by hull addition being due to CT (Yu *et al.* 1995a, 1995b). Addition of hulls significantly lowered the apparent and true ileal digestibility of several essential amino acids in monogastric animals, with part of the effect being attributable to CT (Yu *et al.* 1995c).

All of these studies were done with unheated solvent extracted cottonseed kernel as the source of protein. However, following oil extraction in commercial CSM processing, the meal is heated at 110°C for 2 h to remove residual hexane solvent (M. Mittasch, personal communication). Therefore, it is necessary to determine if the effects of CT which have been established with unheated cottonseed kernels also occur when the kernels are heated under similar conditions to those used during the manufacture of CSM. Objectives of the present study were to determine the quantitative effects of heat treatment on the chemical composition of CSM, with or without added cottonseed hulls (containing CT) and upon the reactivity of CT. Effects measured included available lysine content, *in vitro* total nitrogen (N) solubility and *in vitro* rumen degradation of two individual kernel proteins (52 and 48 kDa), as these parameters are likely to be most affected by heat. The 52 and 48 kDa proteins are the two principal storage proteins, and storage proteins comprise about 70% of the total protein in cottonseed kernels (Dure and Chlan 1981).

7. 3 MATERIALS AND METHODS

7. 3. 1 Experiment Design

An experiment was conducted to determine the effect of heat treatment upon the chemical composition of CSM and upon the reactivity of cottonseed CT. Whole cottonseed was manually separated into hulls and kernels. Oil was extracted from the kernels and both kernels and hulls were then fine ground and prepared as a number of mixtures with defined contents of hulls to kernels. All investigations were done with seeds of *Gossypium hirsutum*, var. Siokra L22, as supplied by Cotton Seed Distributors Ltd, Wee Waa, NSW, Australia. The treatments were cottonseed kernel plus either 0, 25 or 50% hulls (ie 100 g + either 0, 25 or 50 g hulls). In addition, representative samples of commercial CSM produced from Australian's two largest plants (Brisbane and Narrabri) were also analysed.

The effects of heat treatment were determined by comparing solvent extracted samples either with or without heat treatment. The temperature and duration of heating used were similar to those used in the commercial manufacture of CSM. The effect of CT was assessed by determining N solubility and rumen degradation of cottonseed kernel protein in the presence or absence of polyethylene glycol (PEG, molecular weight (MW) 3,500, Union Carbide, Danbury, CT, USA). PEG binds strongly to CT and can be used to displace protein from the CT-protein complexes (Jones and Mangan 1977; Barry and Manley 1986). Therefore, effects of CT can be quantified by comparing controls (CT acting) with PEG treatments (CT inactivated). In the present work PEG was added at a ratio of 2 mg mg^{-1} total CT to maximise displacement of protein from the CT-protein complexes (Yu *et al.* 1995b).

7. 3. 2 Preparation of Cottonseed Samples

Delinted whole cottonseed was cracked by passage through a Crushing-Mill (AB Thorell & Persson, Uppsala, Sweden), and then separated into kernels and hulls using an air-blow technique, as described by Yu *et al.* (1995a). Separated kernels were freeze-dried for 2 days, and then ground to pass through a 2 mm diameter sieve. Ground kernels were extracted in 1 kg batches by continuous stirring with pure hexane (1:2 w/v) for 6 h at ambient temperature, according to the method of Pons and Eaves (1967). After displacement washing with hexane (5 × 1,000 ml), using vacuum filtration, the extracted meal was air-dried, and ground to pass through a 1 mm sieve. Separated hulls were ground to pass through a 1 mm diameter sieve. Extracted cottonseed kernel and ground hulls were stored at -20°C until being used.

The commercial CSM samples from the Narrabri and the Brisbane plants of Cargill Oilseeds Ltd, Australia were produced using the pre-press solvent (hexane) extraction method to remove oil. One random sample of meal was taken daily for 4 days, to provide 4 representative samples from each plant for analysis. All samples were ground to pass through a 1 mm diameter sieve and stored at -20°C until chemical analysis.

7. 3. 3 Experimental Heat Treatment

Separate 1 kg batches of solvent extracted cottonseed kernels were mixed with 0, 250 and 500 g ground cottonseed hulls. Four replicates of each treatment were prepared. The resulting mixtures were then divided into two samples of about 500 g each. One of the two samples was heated for 2 h in a forced-draught oven (100°C; atmospheric pressure; 90 g kg^{-1} moisture at starting time) in flat pans at a thickness of about 1 cm of meal. The second sample was unheated. Chemical analysis of the four replicates per treatment were then carried out as described bellow.

7. 3. 4 *In Vitro* Nitrogen Solubility and Rumen Protein Degradation

In vitro solubility of the total nitrogen (N) in each sample (from 4 replicates per treatment) before and after heat treatment was determined in duplicate in phosphate buffer (pH 7.0) with or without PEG addition. Phosphate buffer was freshly prepared by mixing 195 ml of 0.15 M NaH_2PO_4 with 305 ml 0.15 M Na_2HPO_4 and making up to 1 litre with distilled water. Fifty ml of the buffer and 1 g of sample were added to groups of volumetric flasks (250 ml). At each level of hull treatment, PEG was added to 4 of the 8 flasks per treatment. Flasks were fitted with Bunsen valves and incubated in a shaking water bath (90 rpm) at 39°C for 2 h. The details of *in vitro* incubation have been described by Yu *et al.* (1995a).

Rumen fluid for *in vitro* protein degradation studies was collected from two wether sheep. They were fitted with a rumen cannula (55 mm ID) and were maintained on a basal diet of 800 g d^{-1} meadow hay (890 g kg^{-1} DM, 20 g kg^{-1} N) supplemented with 125 g d^{-1} commercial cottonseed kernels (950 g kg^{-1} DM, 55 g kg^{-1} N, 2.3 g kg^{-1} total CT) which were unextracted and contained approximately 5% hulls (Yu *et al.* 1993). Feed was offered at hourly intervals from overhead belt-feeders for at least 2 weeks prior to each experiment commencing. Rumen fluid from the two sheep was collected at 08:00 h after overnight fasting (8 hours), and pooled rumen fluid was maintained at 39°C under an atmosphere of CO_2.

Freshly prepared artificial saliva (60 ml, pH 6.8; McDougall 1948), rumen fluid (15 ml) and 150 mg of cellobiose (Sigma, St Louis, USA) were added to each volumetric flask (250 ml). Each flask was then flushed with CO_2 and 1 g of each sample was added. PEG was added to four of the eight flasks at each level of hull treatment. The flasks were fitted with a Bunsen valve and shaken (90 min^{-1}) at 39°C for 24 hours. Aliquots (475 µl) were removed from each flask after 0, 0.5, 1, 2, 4, 8, 12, 16 and 24 h of incubation, and added to Eppendorf tubes containing 25 µl of 10% sodium dodecyl sulphate (SDS), as described by Yu *et al.* (1995b).

Rumen degradability of the two main storage proteins (52 and 48 kDa) in cottonseed kernel was measured by fractionating proteins in rumen incubation samples using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then quantifying the individual proteins using imaging densitometry (Bio-Rad, Model GS-670 Imaging Densitometer, USA), as described by Yu *et al.* (1995b).

7. 3. 5 Chemical Analysis

Total lysine content was determined in duplicate 10 mg samples using high performance liquid chromatography (HPLC, Waters Associates, USA). Samples were hydrolysed in 500 µl of 6M HCl with 1% added phenol, for 24 hours at 110±1°C in glass tubes sealed under vacuum. The free amino acid molecular weight was used to calculate the weight of lysine. Available lysine content was determined by the method of Carpenter (1960) as revised by Booth (1971) in which the test materials were treated with 2,4-dinitrofluorobenzene (FDNB), hydrolysed with acid and the resulting mono-ε-*N*-dinitrophenyl-lysine (DNP-L) was measured colorimetrically.

In this paper, total lysine is defined as the lysine content of the unheated original cottonseed materials determined by conventional amino acid analysis. Available lysine is defined as that reacting with FDNB in unheated and heated cottonseed meals. Total lysine values for heated cottonseed materials were not determined, because during the acid hydrolysis step of conventional amino acid analysis a proportion of the heat-induced Maillard compounds revert back to lysine and therefore this leads to an overestimate of the amount of lysine present in heated materials (Moughan *et al.* 1995).

The CT contents of each sample were determined using the method of Terrill *et al.* (1992). Free gossypol in the mixtures was estimated by the method Ba 7-58 of AOCS (1975). Samples of each treatment were also taken for analysis of dry matter (DM) and total N (Kjeldahl procedure) content before and after heat treatment.

7. 3. 6 Data Analysis

Data obtained from the present study were subjected to factorial analysis of variance (ANOVA). Significant differences between the treatment groups were determined by the least significant difference (LSD) test (Steel and Torrie 1980).

In the *in vitro* protein degradation studies, the disappearance of each protein, represented by change in density of the protein bands on the gels, were plotted against time of incubation. The *in vitro* degradation of each protein in the samples during the incubation with rumen fluid was calculated using the following equation (Ørskov and McDonald 1979).

$$Y = A+B\ (1-e^{-Ct}) \qquad (1)$$

where Y represents percent degradation of the 52 and 48 kDa proteins in the rumen at time (t). The constants A, B and C represent, respectively, the instantly soluble fraction (A), the proportion degraded in time t (B) and the degradation rate of the 'B' fraction (C). The constants A, B and C for each animal were calculated by using NLIN (non-linear regression) procedures (SAS 1985). The mean values for 4 replicates of each treatment was used in each of these NLIN calculations, as values for individual replicates showed considerable random variation. Potential degradability was calculated as constants $A+B$.

7.4 RESULTS

7.4.1 Laboratory Samples

Addition of hulls to kernels diluted the contents of total N and free gossypol, but increased total CT content (Table 7. 1). Heating pure cottonseed kernel and mixtures of kernels and hulls did not affect total N concentration, but decreased free gossypol and the content of total CT which reacted with butanol/HCl by 25-27% and 12-14%, respectively.

Addition of hulls to kernels substantially reduced the concentrations of total lysine and of FDNB available lysine, when expressed both as g kg^{-1} DM and as a percentage of total lysine or of crude protein ($p<0.001$; Table 7. 2). Heat treatment further reduced the content of FDNB available lysine, when expressed both as g kg^{-1} DM and as a percentage of crude protein, with the effects being statistically significant ($p<0.001$) but of small magnitude. There was no statistically significant interaction between the addition of hulls and heat on FDNB available lysine content.

In the absence of hulls, 49% of the total N in cottonseed kernel was soluble in mineral buffer (Table 7. 2). This progressively declined ($p<0.001$) as increasing quantities of hulls were added to the kernel. When heat treatment was applied to these

samples, the solubility of total N was further depressed ($p<0.001$). The addition of PEG increased total N solubility ($p<0.001$), and there was a significant interaction ($p<0.001$) between hulls and PEG, explained by PEG addition, which inactivates CT, increasing solubility in the presence of hulls but not in the absence of hulls. Although PEG addition did increase N solubility in the presence of hulls, values did not increase to the same level as that found for pure cottonseed kernel, and responses were of similar magnitude in heated and unheated CSM.

Table 7. 1 Chemical composition[1] (g kg^{-1} DM) of the unheated and heated solvent-extracted cottonseed kernel, mixtures of kernel and hulls, and commercial cottonseed meal (CSM)

Treatment	Dry matter	Total nitrogen	Free gossypol	Condensed tannin[2]			
				Extract-able	Protein-bound	Fibre-bound	Total
Laboratory samples:							
Cottonseed kernel (CSK)	910	75.5	19.1	ND	ND	ND	ND
Heated CSK	988	74.8	14.2	ND	ND	ND	ND
CSK+25% hulls	907	61.8	15.4	2.9	4.0	3.0	9.9
Heated CSK+25% heated hulls	989	62.1	11.3	1.9	4.3	2.4	8.5
CSK+50% hulls	904	53.2	12.6	5.0	7.0	5.3	17.3
Heated CSK+50% heated hulls	989	53.1	9.5	6.0	6.1	3.1	15.2
Commercial samples[3]:							
Commercial CSM Brisbane	917	71.2	0.6	0.6	3.1	2.0	5.7
Commercial CSM Narrabri	910	66.0	0.6	1.8	5.2	2.1	9.1

1 Mean of four replicate determinations.

2 ND; not determined. CT is present in hulls, but not in kernels (Yu *et al.* 1993).

3 Commercial CSM Brisbane was from Brisbane cottonseed processing plant, QLD, Australia; Commercial CSM Narrabri was from Narrabri cottonseed processing plant, NSW, Australia.

In vitro degradation of both the 52 and 48 kDa proteins incubated with rumen fluid was very rapid, with most of the potentially degradable proteins disappearing during the initial 4 h period. This is shown in Figure 1 for the 52 kDa protein. Degradation rate (constant *C*) was rather variable and not affected by the treatments applied (Table 7. 3), whilst potential degradability (constants *A*+*B*) of both the 52 and 48 kDa cottonseed kernel proteins was reduced by both the addition of hulls and the application of heat. However, it was not affected by the addition of PEG to pure cottonseed kernels but was increased by PEG at the high rate of hull addition, with effects at the low rate of hull addition being intermediate. The effects of PEG were similar in heated and unheated materials, and the increase in potential degradability induced by PEG addition was still less than potential degradability of kernel proteins in

the absence of hulls. The effects of PEG addition, in the presence and absence of hulls, for the 52 kDa protein are shown in Figure 7. 1.

Table 7. 2 Effect of heat treatment, addition of hulls and/or polyethylene glycol (PEG)[1] upon FDNB available lysine content[2] and *in vitro* nitrogen solubility[3] of cottonseed kernel (CSK), mixtures of kernel and hulls, and commercial cottonseed meal (CSM)

	Total	Available lysine			Nitrogen	
	lysine	(g kg^{-1}	(% of total	(% of	solubility (%)	
Treatment	(g kg^{-1} DM)	DM)	lysine)	protein)	PEG-	PEG+
Laboratory samples:						
Cottonseed kernel	10.9	10.1	92	2.14	49	50
Heated CSK	-	9.4	-	2.01	45	47
CSK+25% hulls	8.7	6.8	78	1.75	42	46
Heated CSK+25% heated hulls	-	6.4	-	1.64	39	43
CSK+50% hulls	7.5	4.8	64	1.45	37	46
Heated CSK+50% heated hulls	-	4.6	-	1.39	35	39
SEM	0.10	0.09	1.39	0.023	0.38	0.43
Commercial samples[4]:						
CSM Brisbane	9.4	7.8	-	1.76	18	18
CSM Narrabri	9.7	7.0	-	1.70	33	37
SEM	0.06	0.12	-	0.027	0.80	0.64

1 Polyethylene glycol, MW 3,500; PEG-, no PEG added to incubation; PEG+, PEG added to incubation.

2 Measured by fluorodinitrobenzene (FDNB) reactivity (Booth 1971).

3 Nitrogen soluble in phosphate buffer (pH 7.0, incubated at 39°C for 2 h; Yu *et al.* 1995a).

4 Commercial CSM Brisbane was from Brisbane cottonseed processing plant, QLD, Australia; Commercial CSM Narrabri was from Narrabri cottonseed processing plant, NSW, Australia.

7. 4. 2 Commercial Samples

CSM from Brisbane had a greater crude protein (total N × 6.25) content and a lower total CT content than CSM from Narrabri (Table 7. 1). Only 10-11% of total CT in both meals was extractable with a mixture of acetone/water/diethyl ether (4.7:2.0:3.3 v/v), with the remainder being bound to either protein or fibre. Free gossypol content of both meals was around 0.6 g kg^{-1} DM.

FDNB available lysine content (g kg^{-1} DM) was higher in the Brisbane meal than in the Narrabri meal ($p<0.01$; Table 7. 2), but there was no significant difference between the two meals when FDNB available lysine was expressed as a percentage of crude protein. *In vitro* total N solubility of Brisbane CSM in phosphate buffer (pH 7.0) was 18%, substantially lower than that of Narrabri CSM ($p<0.001$; Table 7. 2). There was a significant source of meal×PEG interaction ($p<0.5$), with addition of PEG not

affecting N solubility of Brisbane meal, but significantly increasing N solubility of meal produced at Narrabri.

Table 7. 3 Effect of heat treatment, and/or adding cottonseed hulls and polyethylene glycol (PEG)[1] upon the degradation[2] of cottonseed kernel proteins during *in vitro* incubation with rumen fluid[3]

	52-kDa protein				48-kDa protein			
	Degradation rate C (% h^{-1})		Potential degradability A+B (%)		Degradation rate C (% h^{-1})		Potential degradability A+B (%)	
	PEG-	PEG+	PEG-	PEG+	PEG-	PEG+	PEG-	PEG+
Laboratory samples:								
Cottonseed kernel (CSK)	0.90	1.14	79	79	1.02	1.11	80	83
Heated CSK	1.18	0.96	70	68	1.22	0.93	74	69
CSK+25% hulls	1.01	0.65	73	71	1.17	0.96	75	76
Heated CSK+25% heated hulls	0.86	1.05	71	73	0.87	0.75	71	75
CSK+50% hulls	0.91	0.56	64	72	0.73	1.03	68	71
Heated CSK+50% heated hulls	0.53	1.34	51	58	0.90	1.13	56	66
Commercial samples[4]:								
CSM Brisbane	0.07	0.07	83	86	0.07	0.11	81	92
CSM Narrabri	0.16	0.14	85	90	0.15	0.15	85	91

1 Polyethylene glycol, MW 3,500; PEG-, no PEG added to incubation; PEG+, PEG added to incubation.

2 Mean of four replicate determinations. Protein degradation (Y; %) expressed as a percentage of that present at t=0, was fitted to the equation (Ørskov and McDonald 1979): $Y = A + B\ (1 - e^{-Ct})$, where A, B and C are constants, and t is the time (h) of incubation with rumen fluid.

3 Fifteen ml of rumen fluid, 60 ml of artificial saliva, 150 mg of cellobiose (Sigma, St Louis, USA) and 1 g of either heated or unheated cottonseed products were incubated at 39°C for 24 h. Rumen fluid was collected from sheep fed on 800 g.d^{-1} meadow hay and 125 g d^{-1} commercial cottonseed kernel.

4 Commercial CSM Brisbane was from Brisbane cottonseed processing plant, QLD, Australia; Commercial CSM Narrabri was from Narrabri cottonseed processing plant, NSW, Australia.

Degradation patterns of the 52 and 48 kDa proteins in both meals are shown in Figure 7. 2. The potentially degradable proteins in both meals disappeared slowly, with most degradation being completed by 16 h of incubation. Addition of PEG to the meals during *in vitro* incubation with rumen fluid appeared to increase potential degradability of the 52 and 48 kDa proteins in both meals (Table 7. 3). Degradation rate (constant C) for both proteins in Brisbane meal was considerably lower than those of Narrabri meal. PEG addition did not affect the degradation rate of the two proteins in either meal.

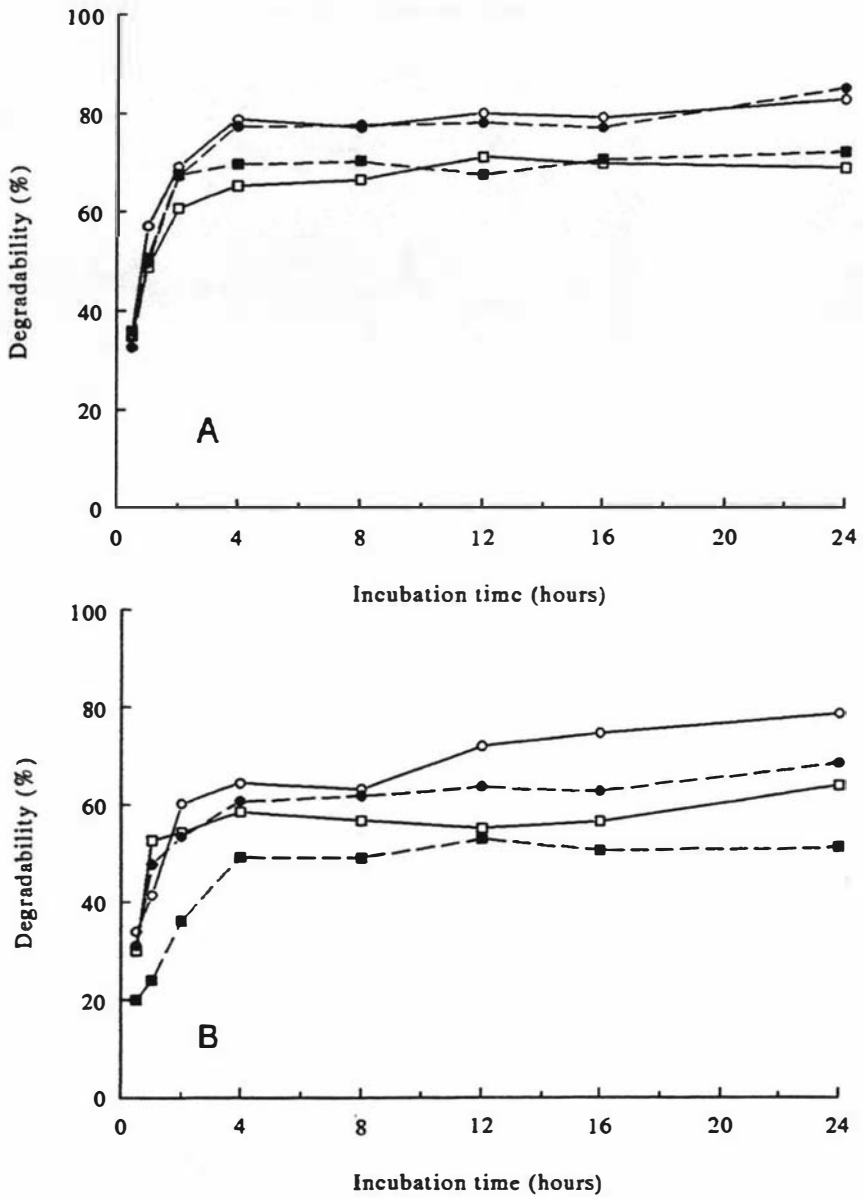

Fig 7. 1 Degradation of the 52 kDa protein of cottonseed kernel (CSK; A) and CSK+50% hulls (B) with or without heat treatment during the *in vitro* incubation in rumen fluid. Fifteen ml of strained rumen fluid, 60 ml of artificial saliva and 1 g of either CSK or CSK+50% hulls were incubated at 39^{o}C for 24h. ●, CSK (A) and CSK+50% hulls (B); ○, CSK+PEG (A) and CSK+50% hulls+PEG (B); ■, heated CSK (A) and heated CSK+50% heated hulls (B); □, heated CSK+PEG (A) and heated CSK+50% heated hulls+PEG. Each point is the mean value of four replicates for each treatment in duplicate determinations.

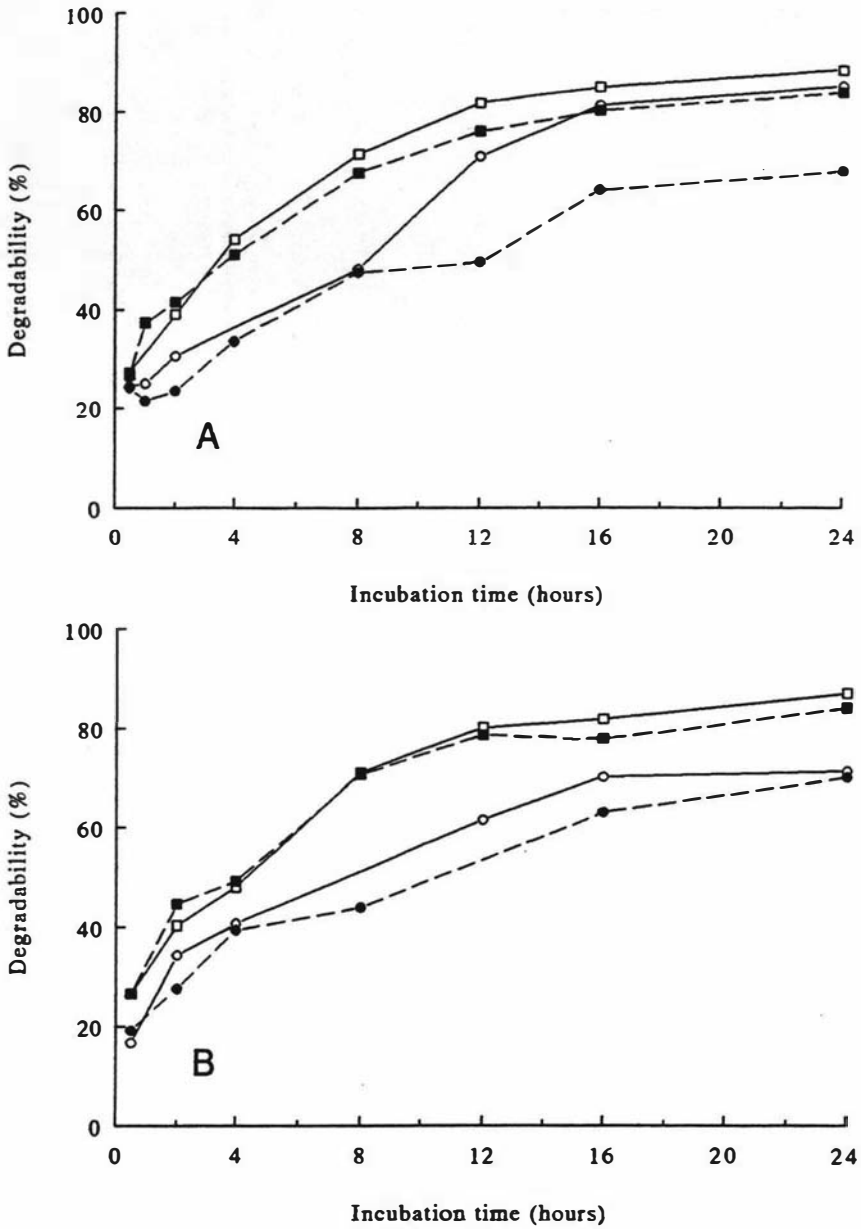

Fig. 7. 2 Degradation of the 48 kDa protein (A) and 52 kDa protein (B) of commercial cottonseed meals (CSM) during the *in vitro* incubation in rumen fluid. Fifteen ml of strained rumen fluid, 60 ml of artificial saliva and 1 g of CSM were incubated at 39°C for 24h. ●, CSM Brisbane; ○, CSM Brisbane+PEG; ■, CSM Narrabri; □, CSM Narrabri+PEG. Each point is the mean value of four replicates for each CSM in duplicate determinations.

7. 5 DISCUSSION

Major findings of this study were that heat treatment caused small but statistically significant reductions in the concentrations of free gossypol and FDNB available lysine, reduced the solubility of total N, and reduced potential degradability of the 52 and 48 kDa cottonseed storage proteins by mixed rumen micro-organisms. The effects of heat treatment which lead to reduce protein digestion have been extensively reviewed by Erbersdobler (1976) and Hurrell and Carpenter (1977). In addition to the effects of sugar-protein Maillard reactions (Adrian 1974) and isopeptide cross-links (Hurrell *et al.* 1976), it is well known that gossypol, when released from cottonseed pigment glands, can react with protein amino groups (Frank 1987). Gossypol contains two carbonyl groups per molecule and hence can effectively cross-link peptide chains (Lyman *et al.* 1959), mainly through reaction with the ε-amino group of lysine. Craig and Broderick (1981) reported that heating CSM by autoclaving caused stepwise increases in binding of free gossypol. In the present studies, heat treating CSM in a forced-draught oven for 2 h (100°C; atmospheric pressure) reduced the contents of both free gossypol and FDNB available lysine.

Variation in amino acid composition of CSM is due principally to changes in total lysine and available lysine contents (Batterham 1992). Screw-pressed meals, which are subjected to the most heating, are generally lowest in total and available lysine (Jones 1981). The loss of available lysine in CSM is due partly to its reaction with gossypol during processing, but other compounds, such as sugars (Martinez *et al.* 1967) and oxidised fats (Carpenter and Booth 1973; Erbersdobler 1976), are also involved. Some previous workers have found close agreement between FDNB-available lysine and the protein efficiency ratio of CSM (Martinez *et al.* 1967; Craig and Broderick 1981) and growth of rats fed heat-damaged meat proteins (Boctor and Harper 1968). However, others have not found a strong correlation between FDNB-available lysine and nutritionally available lysine in CSM (Batterham *et al.* 1979).

Finot and Mauron (1972) showed that even α-*N*-formyl-(ε-*N*-deoxyfructosyl)-lysine (FFL), a pure Maillard compound which has no nutritional value as a source of lysine, can give approximately a 50% yield of lysine on acid-hydrolysis. The total lysine values obtained with commercial CSM in this study may, therefore, be overestimations due to some Maillard products breaking down to give free lysine during acid hydrolysis (Frangne and Adrian 1972; Moughan *et al.* 1995).

In this study, both total N solubility and potential degradability of cottonseed proteins were reduced by heating. Broderick and Craig (1980) suggested that heat treatment decreased ruminal degradation partly by blocking reactive sites for microbial

proteolytic enzymes and partly by reducing protein solubility; the present results partly support this concept.

The results obtained from the present study show that heat-treating mixtures of hulls and cottonseed kernel reduced the concentration of total CT that could be extracted with acetone/water and SDS solution and determined with butanol/HCl by 12-14%. Total CT concentration in the commercial CSM samples that reacted with butanol/HCl was also reduced (by 35-40%) compared with previous determinations on the same materials two years earlier (Yu *et al.* 1993). Exposure to oxygen (including drying) can cause oxidative damage to CT (Goldstein and Swain 1965; McLeod 1974), and the above results may most likely be explained by the oxidation of some CT to other compounds that are not detected by butanol/HCl.

The *in vitro* studies showed that adding hulls depressed solubility of total N and depressed ruminal degradation of the two major storage proteins (52 and 48 kDa) in cottonseed kernel. However, the *in vitro* PEG data indicated that only part of the depression could be explained by the presence of CT in the hulls. Similar results have been reported by Yu *et al.* (1995a, 1995b). It seems that the remainder of the hull effect must be due to insoluble protein in hulls and other unknown components present in hulls. As the effect of PEG in reversing the effect of CT was of similar magnitude in heated as in unheated CSM, it seems that heating did not reduce the reactivity of CT with cottonseed proteins.

An unexpected and potentially very important result obtained in the present study was the effect of hull addition in reducing FDNB available lysine content (% total lysine) in the unheated CSM. The cause is unknown, but may involve cottonseed CT. If confirmed in further work, this would seem to be an important anti-nutritional property of cottonseed hulls and further indicates why the content of hulls should be minimised in CSM intended for consumption by monogastric animals.

Commercial CSM used in this experiment had lower levels of free gossypol, N solubility and rumen protein degradation rate (constant *C*) compared with laboratory prepared cottonseed samples, indicating that commercial processing had caused more pronounced changes in chemical reactivity than our laboratory procedures. The temperature (100°C) and time of heating (2 h) used in the laboratory studies were similar to those used in CSM processing at Narrabri, Australia, but moisture contents differed between the two procedures. Moisture is kept within the range 7-8% during initial heating by injection of water and at 11-17% during final heating by injection of steam in CSM produced at Narrabri, which can be defined as wet heat, whereas our forced draught oven drying used dry heat. Tagari *et al.* (1986) concluded that autoclaving at 120°C (wet heat) was more effective than dry heat at 120°C for reducing

rumen degradation of N in cottonseed. The moisture and pressure used in commercial processing probably enhanced the heating process, making it more effective than dry heat at a given temperature. Moisture content should therefore be included as a factor in future heating studies.

CSM produced from the Brisbane mill had lower N solubility and lower ruminal protein degradation rate than CSM produced from the Narrabri mill, suggesting that greater heat had been generated in processing at Brisbane. This would have been expected to reduce lysine availability, but interestingly the FDNB available lysine content (% crude protein) was similar in the CSM produced by the two mills. An explanation might be the lower hulls and hence lower CT content of CSM produced at Brisbane. Yu *et al.* (1996) have shown that the effects of cottonseed CT in reducing amino acid digestion in monogastric animals are greater in heated than in unheated CSM for threonine, tyrosine and lysine, but that the depression for all other amino acids is similar in heated and unheated materials. The lower hull (and hence CT) content of Brisbane CSM may have minimised heat damage to lysine. Interactions among heating temperature, heating time, moisture content, hulls and CT need to be studied in future work. Also, not all reactive lysine (FDNB) is necessarily absorbed and the effects of CT and heat should be studied by determining digestible reactive lysine levels (Moughan and Rutherfurd 1995).

It can be concluded that heating CSM reduced the contents of free gossypol and FDNB available lysine by small but statistically significant amounts, and reduced both N solubility and the degradation of cottonseed proteins by rumen micro-organisms. Addition of hulls further depressed both N solubility and rumen degradation of cottonseed proteins, and as the responses to PEG were similar for both unheated and heated preparations it seems that heat did not diminish the reversible reactivity of CT with cottonseed kernel proteins. Although application of heat inactivated 13% of the total CT such that it could no longer be extracted and detected with butanol/HCl, it did not seem to change the overall effects produced by CT in reducing N solubility and protein degradation.

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Chapter 8

THE EFFECT OF CONDENSED TANNINS FROM HEATED AND UNHEATED COTTONSEED ON THE ILEAL DIGESTIBILITY OF AMINO ACIDS FOR THE GROWING RAT AND PIG

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8. 1 ABSTRACT

The effect of condensed tannins (CT) from heated and unheated cottonseed on the apparent ileal digestibility of amino acids for the growing rat and pig was determined. In Experiment 1, twenty-four rats were allocated to four semi-synthetic diets, which contained cottonseed kernel/hulls as the sole protein source. Two of the diets contained unheated solvent extracted cottonseed kernel/hulls while the remaining two diets contained similar material but which had been heat-treated by autoclaving at 110°C for 120 min. In Experiment 2, twelve rats and twelve pigs were fed four semi-synthetic diets containing commercial cottonseed meal (CSM) as the sole protein source. Chromic oxide was added to all diets as an indigestible marker. For each pair of diets in both experiments, polyethylene glycol (PEG) was either included or excluded. The effect of CT was assessed by comparing control animals (-PEG; CT acting) with PEG supplemented animals (+PEG; CT inactivated). Ileal contents from the terminal 15 and 45 cm of ileum were collected at slaughter, 7 h from the start of feeding, for the rats and pigs, respectively. Apparent ileal amino acid digestibility for rats fed the cottonseed kernel/hulls diet was significantly depressed by the heat treatment, particularly for lysine and threonine. On average, apparent ileal amino acid digestibility in the diets without PEG was decreased from 0.80 to 0.70 by heat treatment. Dietary cottonseed CT depressed apparent ileal protein digestibility in the pig and in the rat. The addition of PEG to the diets significantly increased the apparent ileal digestibility of N and some amino acids for the pigs and the rats. The mean increase in apparent ileal digestibility due to PEG addition for the 14 amino acids was 2% units in both species fed the commercial CSM diets, and 2 or 4% units in rats fed the unheated or the heated cottonseed kernel/hull diets, respectively. The effect of PEG was similar in the heated and unheated cottonseed kernel/hulls for most amino acids, but apparent ileal digestibility of threonine, tyrosine and lysine were increased more by PEG in heated than in unheated CSM. Apparent ileal N digestibility was lower in the pig than in the rat. For several of the amino acids there were significant animal species differences in apparent ileal digestibility. Studies into the effects of cottonseed CT should be carried out in the target animal species. The commercial CSM had a low apparent ileal amino acid digestibility overall, particularly for the essential amino acids lysine and threonine. It was concluded that effects of heating did not eliminate the reversible reactivity of cottonseed CT on amino acid digestion in rats and pigs but rather appeared to increase it for threonine, tyrosine and lysine in Experiment 1, causing large reductions in apparent ileal digestibility of these amino acids.

8. 2 INTRODUCTION

Cottonseed meal (CSM) is commonly used as a protein supplement in the formulation of pig diets, but its nutritive value is lower than that predicted on the basis of its chemical composition (Batterham *et al.* 1990). This is thought to be due to the effects of the processing of cottonseed during oil extraction, and to the presence of several anti-nutritional factors (ANFs), such as gossypol and condensed tannins (CT; Terrill *et al.* 1992; Yu *et al.* 1993). The heating of CSM during commercial processing binds free gossypol to the ε-amino group of lysine (Berardi and Goldblatt 1980) but may also cause other detrimental effects (Batterham 1992). Free amino groups and mainly the ε-amino group of lysine, may react with other groups to form enzyme-resistant inter- and intra-molecular bonds. The solubility and digestibility of the protein is thereby reduced and the time-course of digestion is prolonged (Ford and Shorrock 1971). Condensed tannins are found in cottonseed hulls, but not in cottonseed kernel (Yu *et al.* 1993). Dietary CT have been shown to reduce body weight gain, impair feed conversion efficiency and reduce the apparent digestibility of protein and amino acids in monogastric species (Longstaff and McNab 1991; Jansman 1993; Yu *et al.* 1995b). Our previous work with the growing rat has shown that the addition of cottonseed hulls to a semi-synthetic diet significantly increased endogenous ileal amino acid loss (Yu *et al.* 1995a) whilst hulls, and partly due to their CT content, decreased the apparent and true ileal digestibility of amino acids in cottonseed kernel (Yu *et al.* 1995b). The latter study used unheated solvent extracted cottonseed kernel as the major dietary protein source, and there is a need to establish if the latter finding also holds for heat-treated materials. High temperatures are used in the manufacture of CSM.

In rats, the consumption of high-tannin sorghums, or high-tannin faba bean hulls increased the relative weight of the parotid glands and there was an increased synthesis and secretion of proline-rich proteins with a high affinity for binding tannins (Mehansho *et al.* 1983; Jansman 1993), resulting in a higher endogenous nitrogen excretion in the faeces. The existence of a similar mechanism in pigs fed tannin-rich faba bean hulls has not been demonstrated (Jansman 1993). The quantitative effects of CT in CSM on the apparent ileal digestibility of protein and amino acids in pigs have not been determined.

The objectives of the present study were firstly to determine if the effects of CT in unheated CSK on apparent ileal amino acid digestibility also hold for heat treated CSK, secondly, to quantitatively determine the effect of cottonseed CT on the apparent ileal digestibility of protein and amino acids in the growing pig and thirdly to compare the effects of CT in CSM on the apparent ileal digestibility of protein and amino acids in growing rats and pigs.

The effect of heat treatment on protein digestibility was determined by comparing the digestion of a cottonseed kernel and hulls mixture with or without heat-treatment, with the temperature and duration of heating being similar to those used in commercial processing. The effect of CT was assessed by determining the digestibility of nutrients in the presence or absence of polyethylene glycol (PEG; molecular weight (MW) 3,500, Union Carbide, Danbury, CT, USA). PEG binds strongly to CT and can be used to completely displace protein from CT-protein complexes (Jones and Mangan 1977). The effects of CT can be quantified by comparing animals receiving a control diet (CT acting) with animals given the same diet but with PEG added (CT inactivated). In the present work PEG was added at a ratio of 2 mg mg^{-1} total CT to maximise displacement of protein from the CT-protein complexes (Yu *et al.* 1995c). Previous studies (Yu *et al.* 1995a, 1995b) have established that adding PEG to the diet in the absence of CT has no effect on protein digestion.

8. 3 MATERIALS AND METHODS

8. 3. 1 Preparation of Cottonseed Products

8. 3. 1. 1 Experiment 1

Delinted whole cottonseed (var. Siokra L22) supplied by Cottonseed Distributors Ltd, Wee Waa, NSW, Australia was cracked using a Crushing-Mill (AB Thorell and Persson, Uppsala, Sweden), and separated into kernels and hulls using air-flow, at the Seed Technology Centre, Massey University, with final manual separation. The separated kernels were freeze-dried for 48 h, ground to pass through a 2 mm diameter sieve, and the oil and gossypol were partially extracted using hexane and then acetone in water (70:30 w/w) using a modification of the Pons and Eaves (1967) procedure, as described by Yu *et al.* (1995c). Finally, the extracted cottonseed kernels and hulls were re-ground to pass through a 1 mm diameter sieve and were stored at -20°C.

Three kg of solvent extracted cottonseed kernels were mixed with 500 g ground hulls (166.7g hulls kg^{-1} kernel). The resulting mixtures were then divided into two samples. One of the two samples was heat-treated by autoclaving for 120 minutes (110°C; 0.5 kg $(cm^2)^{-1}$ pressure with 70-100 g kg^{-1} moisture) in flat pans at a thickness of about 2 cm. The second sample remained unheated.

8. 3. 1. 2 Experiment 2

Commercial CSM was supplied by the Narrabri mill of Cargill Oilseeds Ltd, Australia (prepress solvent extraction method, using hexane as the solvent to extract oil) and was sifted through a 1.0 mm screen.

8. 3. 2 Animals and Diets

8. 3. 2. 1 Experiment 1

Twenty-four Sprague-Dawley rats (12 male and 12 female; body weight 178±8.5 g; mean±SD) , which had been weaned at 4 weeks of age, were reared on a high quality diet at the Small Animal Production Unit, Massey University. The animals were kept individually in raised stainless steel cages with wire mesh floors, at 20±2°C and with a 12 h light/dark cycle. Animals were randomly assigned to 4 experimental diets, with 6 animals (3 males and 3 females) on each dietary treatment.

The four semi-synthetic diets were formulated (Table 8. 1) based on corn starch, and contained solvent-extracted cottonseed kernel/hulls as the sole protein source. Chromic oxide was added to all diets as an indigestible marker compound. Two of the diets contained unheated solvent extracted cottonseed kernel and unheated hulls whilst the remaining two diets contained the heated materials. For each pair of diets, PEG was either included or excluded.

8. 3. 2. 2 Experiment 2

Twelve entire-male pigs (body weight 24±1.2 kg; mean±SD), supplied by the Pig Research Unit, Massey University, and 12 male Sprague-Dawley rats (body weight 192±4.5 g; mean±SD), supplied by the Small Animal Production Unit, Massey University, were used as experimental animals. Both the rats and pigs were kept individually in metabolism cages, in a temperature-controlled room (20±2°C) and with a 12 h light/dark cycle. Animals from each species were randomly assigned to 2 experimental diets, with 6 animals on each dietary treatment.

The two semi-synthetic diets were formulated (Table 8. 1) for each species based on corn starch and containing CSM as the sole protein source. Chromic oxide was added as an indigestible marker and PEG was added to one of the diets for each pair to allow the effect of the CT to be distinguished.

8. 3. 3 Experimental Procedure

The animals in both Experiments 1 and 2 were fed a semi-synthetic casein-based preliminary diet for 2 days, after which they were given their respective experimental

Table 8. 1 Ingredient and determined chemical compositions of the solvent-extracted cottonseed kernel/hulls and commercial cottonseed meal (CSM) based diets for the growing rats and pigs

	Experiment 1				Experiment 2			
	Cottonseed kernel/hull diet[1]				CSM diet			
	Unheated		Heated		Rat		Pig	
	PEG-[2]	PEG+[2]	PEG-	PEG+	PEG-	PEG+	PEG-	PEG+
Ingredient (g kg^{-1} air dry)								
Cottonseed kernel	300	300	300	300	-	-	-	-
Cottonseed hulls	50	50	50	50	-	-	-	-
Commercial CSM	-	-	-	-	400	400	400	400
PEG	-	6	-	6	-	12	-	12
Maize starch	444	438	444	438	394	382	394	382
Sucrose	100	100	100	100	100	100	100	100
Maize oil	50	50	50	50	50	50	50	50
Dicalcium phosphate	24	24	24	24	24	24	24	24
Sodium chloride	5	5	5	5	5	5	5	5
Magnesium sulphate	2	2	2	2	2	2	2	2
Potassium carbonate	4	4	4	4	4	4	4	4
Mineral/vitamin premix[3]	15	15	15	15	15	15	15	15
Chromic oxide	6	6	6	6	6	6	6	6
Nutrient (g kg^{-1} DM)[4]								
OM[5]	922	921	924	921	919	921	915	915
Crude protein	175	180	177	183	197	194	202	201
Oil	49	49	44	44	27	31	31	28
NDF[5]	67	66	72	70	54	56	56	56
ADF[5]	35	37	38	39	32	33	33	31
Lignin	18	15	15	12	12	10	11	11
Gross Energy (MJ kg^{-1})	18.7	18.9	18.5	18.7	18.2	18.3	18.1	17.7
Free gossypol (mg kg^{-1})	240	230	160	170	280	260	290	270
Condensed tannin								
Total (calculated)	2.8	2.8	2.0	2.0	4.2	4.2	4.2	4.2

1 Cottonseed kernel (300 g kg^{-1}) + 50 g kg^{-1} hulls.

2 Polyethylene glycol, MW 3,500; PEG-, no PEG in the diet; PEG+, PEG included in the diet.

3 For rat diets: Rat Pellet Premix 9327, Technik Products, Auckland, NZ. For pig diets: Danmix-Pig Grower and Finisher, Nutritech International Ltd, Auckland, NZ.

4 Means of duplicate determinations.

5 OM, organic matter; NDF, neutral detergent fibre; ADF, acid detergent fibre.

diet for 14 days. For the rats, the diets were offered in stainless steel feeders, with the feeder being placed in the cage for 10 min at hourly intervals (0830 to 1630 h). The training was achieved within 7 days. The meal intake of the rats was set at 0.73 of the *ad libitum* digestible energy intake (NRC 1978), and this was sufficient to maintain

substantial growth rates over the trial period. For the pigs, the diets were offered as a wet mash for a single 3-hour period (09.00-12.00 h) for the first 10 days. From day 11 to the last day of study, each pig was fed at hourly intervals (0830 to 1630 h) with equal sized meals. Each meal was mixed with water (1:1; w/v) prior to feeding. The same relative level of meal intake was offered to the pigs as for the rats (0.73 of the *ad libitum* digestible energy intake, equivalent to 0.10 metabolic body weight $kg^{0.75}$ per day). Fresh water was freely available for both species.

On day 14, and 7 hours after the start of the meal, the rats were asphyxiated in carbon dioxide gas and decapitated (immediately ceasing all neural stimulation to the gut), as described by Yu *et al.* (1995b). Ileal digesta were collected from the terminal 15 cm of ileum. The pigs were anaesthetised using halothane gas (Fluothane, Imperial Chemical Industries Ltd, Cheshire, UK) and euthanased by a 20 ml intracardial injection of sodium pentobarbitone (Anathal 60 mg ml^{-1}; VR Laboratories, Thornleigh, NSW, Australia). Digesta were then removed from the terminal 45 cm of ileum. Ileal contents were slowly flushed out using deionized water and were frozen (-20°C) immediately after collection, and then freeze-dried. The stomach contents of the rats were inspected for signs of faecal contamination which results from coprophagy.

8. 3. 4 Chemical Analysis

Prior to analysis, samples of diets and ileal digesta were ground in a laboratory mill with a 1.0 mm screen. The diets and ileal digesta were analysed in duplicate for total nitrogen using the Kjeldahl procedure, and crude protein was calculated as total N × 6.25. The chromium contents of duplicate 20-30 mg samples of ileal digesta and each diet were determined by the method of Costigan and Ellis (1987). The CT contents of the diets were determined using the method of Terrill *et al.* (1992). Free gossypol in the diets was estimated by the method Ba 7-58 of AOCS (1975). The neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin contents were determined by the method of Robertson and van Soest (1981). The crude ash, crude oil and gross energy contents of the feeds were analysed according to conventional methods (AOAC 1975).

Amino acid composition was determined on 10 mg samples using high performance liquid chromatography (HPLC, Waters Associates, USA). Duplicate samples were hydrolysed in 500 µl of 6M HCl with 1% added phenol, for 24 hours at 110±1°C in glass tubes sealed under vacuum. The amino acids, methionine, cystine and tryptophan, which were partly destroyed during acid hydrolysis, were not determined. Free amino acid molecular weights were used to calculate the weights of the amino acids.

8. 3. 5 Data Analysis

Apparent digestibility coefficients for dry matter, protein and amino acids were calculated using the following equation (Maynard *et al.* 1979):

$$\text{Apparent digestibility (\%)} = 100 - 100 \frac{\text{\% chromium in feed}}{\text{\% chromium in ileal digesta}} \times \frac{\text{\% nutrient in ileal digesta}}{\text{\% nutrient in feed}}$$

A linear statistical model, which included terms for heat, PEG and heat × PEG for Experiment 1 and animal species, PEG and species × PEG for Experiment 2, was fitted to the digestibility data for dry matter, protein and each amino acid singly, and reduction in sums of squares was used to determine levels of significance (Steel and Torrie 1980).

8. 4 RESULTS

8. 4. 1 Chemical Composition

The determined chemical composition of the cottonseed kernel, hulls and their mixture, before and after heat-treatment, and the commercial CSM is shown in Table 8. 2. Protein and fibre (NDF) contents in the cottonseed kernel were 545 and 80 g kg^{-1}, and in the commercial CSM were 498 and 128 g kg^{-1}. Free gossypol content was lower in the kernel compared to commercial CSM (0.6 vs 0.9 g kg^{-1}). Cottonseed hulls contained 45 g protein kg^{-1} DM, 750 g NDF kg^{-1} DM, 0.3 g free gossypol kg^{-1} DM and 46.3 g total CT kg^{-1} DM. Heat treatment of the kernel/hull mixture reduced the free gossypol concentration (29%) and the total CT concentration measured with butanol/HCl (29%), but did not affect the amino acid content.

8. 4. 2 Experiment 1

The mean (±SD) bodyweight of the rats at the end of the study was 222±12.0 g. Mean food intakes for the rats on day 13 of the study are given in Table 8. 3. Food intake was within the normal range for the 220 g bodyweight rat (NRC 1978). Dietary PEG addition did not appear to affect food intake. On the last day of study, the rats had relatively high food intakes over the first two hourly meals and then consumed generally even sized meals for the remainder of the feeding period (Table 8. 3). The latter was important to ensure an even flow of digesta at the terminal ileum. Faeces were not detected in the gastric contents at slaughter indicating that coprophagy had not occurred at least on the last day of study.

Table 8. 2 Chemical compositions[1] (g kg^{-1} DM) of the unheated and heated solvent-extracted cottonseed kernels, cottonseed kernel/hulls, hulls and of the commercial cottonseed meal

	Cottonseed kernel			Cottonseed hulls	Cottonseed meal
	Pure unheated	Unheated+ unheated hulls[2]	Heated+ heated hulls[2]	Pure unheated	
Dry matter (g kg^{-1})	892	892	920	888	878
Crude protein (N×6.25)	545	475	469	45	498
Oil	117	103	97	17	21
Neutral detergent fibre	80	187	191	751	128
Acid detergent fibre	28	100	95	524	76
Lignin	16	45	42	212	26
Free gossypol	0.59	0.48	0.34	0.31	0.94
Condensed tannin[3]					
Extractable	ND	2.38	0.84	13.68	0.65
Protein-bound	ND	4.58	3.88	24.64	8.98
Fibre-bound	ND	0.92	0.90	8.03	0.91
Total (calculated)	ND	7.88	5.62	46.34	10.54
Essential amino acid					
Arginine	32.1	27.0	26.7	1.1	26.2
Histidine	9.9	8.6	8.7	0.7	8.0
Isoleucine	8.5	7.1	7.5	0.6	6.9
Leucine	15.8	13.4	14.0	1.0	12.9
Lysine	12.1	10.3	9.7	1.0	9.7
Phenylalanine	14.6	13.8	12.8	0.8	11.9
Threonine	9.6	8.1	8.4	0.7	7.8
Valine	7.7	9.7	10.2	0.8	9.5
Total (calculated)	110.3	98.0	98.0	6.7	92.9
Non-essential amino acid					
Alanine	10.9	9.2	9.6	0.8	9.0
Aspartic acid	23.7	20.2	20.8	1.6	20.5
Glutamic acid	40.0	39.7	40.5	2.8	40.2
Glycine	11.4	9.8	10.1	0.8	9.5
Serine	11.3	9.7	9.9	1.0	9.2
Tyrosine	8.6	7.1	7.3	0.7	6.6
Total (calculated)	105.9	95.7	98.2	7.7	95.0

[1] Means of duplicate determinations.

[2] Cottonseed kernel (300 g kg^{-1}) + 50 g hulls kg^{-1}.

[3] ND, not determined; CT is present in hulls, but not in kernels (Yu *et al.* 1993).

Table 8. 3 Mean food intakes[1] for the growing rats on day 13 and hourly meal intakes for the last day (day 14) of study in Experiment 1

Diet	Food intake on day 13 (g)	Hourly meal intakes on day 14[2] (g)							
		1	2	3	4	5	6	7	Total
Cottonseed kernel/hulls:									
50 g kg^{-1} hulls	16.3	3.0	2.7	2.1	2.2	2.2	1.5	0.7	14.4
50 g kg^{-1} hulls+PEG[3]	16.3	2.8	2.3	2.0	1.9	2.2	1.5	0.7	13.4
Heated kernel/hulls:									
50 g kg^{-1} hulls	15.6	3.5	3.0	2.0	2.2	2.6	1.0	0.7	15.0
50 g kg^{-1} hulls+PEG	16.5	3.0	2.5	2.0	1.9	2.1	1.2	1.2	13.9

1 Each value is a mean (n=6).
2 Day of sampling ileal digesta.
3 Polyethylene glycol, MW 3,500.

Heat treatment significantly depressed ($p<0.001$) ileal DM digestibility and the apparent ileal digestibility of nitrogen (N; Table 8. 4). Dietary supplementation with PEG significantly increased ileal DM digestibility ($p<0.05$) and apparent ileal N digestibility ($p<0.001$). There was no significant interaction between heat treatment and PEG for either ileal DM digestibility or the apparent ileal digestibility of N.

Apparent ileal amino acid digestibility for rats fed the cottonseed kernel/hulls based diet was significantly depressed by the heat treatment (Table 8. 4) for all amino acids except histidine, and the depression was particularly marked for lysine and threonine. On average, apparent ileal amino acid digestibility in the diets without PEG addition was decreased from 0.80 to 0.70 by the heat treatment. Addition of PEG to either the heated or unheated treatments led to a significant increase in apparent ileal amino acid digestibility. The results indicate that heat treatment had a major effect on decreasing the protein quality of the cottonseed kernel and that CT in the diet further depressed the ileal digestibility of amino acids. Histidine digestibility was not affected by heat treatment nor by PEG addition to the diets.

There were no statistically significant interactions ($p>0.05$) between heat treatment and PEG for apparent amino acid digestibility, except for threonine and tyrosine. The significant heat×PEG interactions for threonine ($p<0.05$) and tyrosine ($p<0.001$) were explained by the responses to PEG being greater on heated than on unheated CSM. A similar trend was evident for lysine, with the interaction attaining significance at $p=0.06$.

Table 8. 4 Mean (n=6) apparent digestibility of dry matter, total nitrogen and amino acids determined at the terminal ileum of the growing rat given a solvent-extracted cottonseed kernel/hulls based diet in Experiment 1

	Cottonseed kernel/hull diet							
	Unheated		Heated		Overall	Level of significance[1]		
	PEG-[2]	PEG+[2]	PEG-	PEG+	SE	Heat	PEG	H×P
Dry matter	0.74	0.76	0.70	0.72	0.008	***	*	NS
Nitrogen	0.79	0.86	0.70	0.74	0.014	***	***	NS
Essential amino acid								
Arginine	0.92	0.93	0.86	0.88	0.007	***	0.08	NS
Histidine	0.88	0.89	0.87	0.87	0.012	NS	NS	NS
Isoleucine	0.75	0.78	0.64	0.68	0.010	***	**	NS
Leucine	0.78	0.80	0.69	0.71	0.010	***	**	NS
Lysine	0.77	0.80	0.58	0.64	0.008	***	***	0.06
Phenylalanine	0.84	0.86	0.78	0.81	0.006	***	**	NS
Threonine	0.69	0.69	0.56	0.62	0.013	***	0.06	*
Valine	0.78	0.80	0.69	0.72	0.009	***	**	NS
Non-essential amino acid								
Alanine	0.77	0.80	0.66	0.71	0.010	***	***	NS
Aspartic acid	0.83	0.85	0.70	0.75	0.014	***	**	NS
Glutamic acid	0.88	0.90	0.79	0.82	0.009	***	**	NS
Glycine	0.73	0.77	0.56	0.61	0.011	***	***	NS
Serine	0.77	0.80	0.67	0.70	0.010	***	**	NS
Tyrosine	0.80	0.82	0.71	0.83	0.008	***	***	***

[1] H×P, interaction between heat and PEG; NS, non significant; *, p<0.05; **, p<0.01; ***, p<0.001.

[2] Polyethylene glycol, MW 3,500; PEG-, no PEG in the diet; PEG+, PEG included in the diet.

8. 4. 3 Experiment Two

The mean (±SD) bodyweights at the end of the study were 242±11.1 g and 29±1.7 kg for the rats and pigs, respectively. All animals consumed the diets readily and were fully accustomed to the feeding procedure after 7 days. On average, the rats and pigs consumed 17.6 g and 1.35 kg of meal, respectively on the day before slaughter. None of the animals used in the experiment showed evidence of ingested faeces in the stomach at slaughter and, therefore, coprophagy was not considered to be of significance.

The apparent ileal digestibility values for DM, N and amino acids for the rats and pigs given the commercial CSM, including or excluding dietary PEG, are given in Table 8. 5. The ileal digestibility of DM and the apparent ileal digestibility of N tended to be lower for the pig than for the rat. Also, for several of the amino acids there were significant species differences in apparent ileal digestibility.

The addition of dietary PEG to the commercial CSM based diets significantly increased the apparent ileal digestibility of N ($p<0.01$) for both the rats and the pigs, but did not affect ileal DM digestibility (Table 8. 5). The effects of PEG addition on apparent ileal amino acid digestibility were similar for the two animal species. Inclusion of dietary PEG led to an increase in the apparent ileal digestibility of the individual amino acids for glycine ($p<0.001$), isoleucine, serine ($p<0.01$), leucine, threonine, valine, alanine, tyrosine, aspartic acid and glutamic acid ($p<0.05$). The apparent ileal digestibility of the remaining amino acids tended to increase with the addition of PEG, but the differences were not statistically significant ($p>0.05$). The mean increase in apparent ileal digestibility for the 14 amino acids, due to the addition of PEG (12 g kg^{-1} DM) to the CSM diet, was 2% units. The species × PEG interaction was not statistically significant, except for the non-essential amino acid, glycine. The commercial CSM had a low apparent ileal amino acid digestibility overall.

Table 8. 5 Mean (n=6) apparent digestibility of dry matter, total nitrogen and amino acids determined at the terminal ileum of the growing rat and pig given a commercial cottonseed meal based diet in Experiment 2

	Cottonseed meal diet							
	Rat		Pig		Overall	Level of significance[1]		
	PEG-[2]	PEG+[2]	PEG-	PEG+	SE	Species	PEG	S×P
Dry matter	0.70	0.70	0.63	0.66	0.011	***	NS	NS
Nitrogen	0.69	0.71	0.66	0.70	0.010	0.08	**	NS
Essential amino acid								
Arginine	0.85	0.87	0.85	0.85	0.006	*	NS	NS
Histidine	0.87	0.87	0.77	0.76	0.012	***	NS	NS
Isoleucine	0.57	0.62	0.61	0.64	0.012	*	**	NS
Leucine	0.62	0.65	0.64	0.66	0.011	NS	*	NS
Lysine	0.50	0.52	0.58	0.59	0.013	***	NS	NS
Phenylalanine	0.76	0.77	0.75	0.78	0.010	NS	0.09	NS
Threonine	0.50	0.53	0.56	0.58	0.009	***	*	NS
Valine	0.63	0.66	0.64	0.67	0.010	NS	*	NS
Non-essential amino acid								
Alanine	0.60	0.63	0.60	0.63	0.012	NS	*	NS
Aspartic acid	0.70	0.71	0.70	0.74	0.010	NS	*	NS
Glutamic acid	0.79	0.81	0.76	0.78	0.007	***	*	NS
Glycine	0.54	0.62	0.60	0.62	0.011	**	***	**
Serine	0.61	0.64	0.63	0.66	0.010	*	**	NS
Tyrosine	0.68	0.69	0.67	0.70	0.008	NS	*	NS

1 S×P, interaction between species and PEG; NS, non significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.
2 Polyethylene glycol, MW 3,500; PEG-, no PEG in the diet; PEG+, PEG included in the diet.

8. 5 DISCUSSION

The present values for apparent ileal amino acid digestibility for the rats fed the solvent-extracted cottonseed kernel and for the pigs fed the commercial CSM are generally similar to previous determinations (Yu *et al.* 1995b) and to other reports in the literature (Taverner *et al.* 1983; Sauer and Ozimek 1986; Baterham *et al.* 1990). The apparent ileal digestibility coefficients for the CSM amino acids were generally low and this was particularly so for lysine, threonine and glycine.

Although the heat treatment used in Experiment 1 appeared to inactivate some 29% of the total CT, which has been observed previously (Yu *et al.* 1996), such that it could not be extracted and detected with butanol/HCl, the heat treatment applied did not reduce the amount of reversible binding by CT as judged by the responses to PEG. Similar results were found by Yu *et al.* (1996) in studying the effect of heat treatment on N solubility and the rumen degradation of cottonseed proteins. Rather, the increased responses to PEG in apparent ileal digestibility of threonine, tyrosine and lysine in heated compared to unheated CSM suggests that action of autoclaving at 110°C for 2 h may in fact have increased the reactivity of CT with these amino acids causing large reductions in digestibility. This clearly requires further study. Action of PEG in Experiment 2 also increased apparent ileal digestibility of many amino acids in both rats and pigs fed commercially produced CSM. However, for the two limiting essential amino acids lysine and threonine, the responses were much less for commercial CSM (1-3% units; Experiment 2) than for heated CSM prepared under laboratory conditions (6% units; Experiment 1), perhaps indicating some irreversible binding under commercial conditions. The commercial CSM was produced by pre-press solvent extraction, and whilst it is possible to duplicate in the laboratory the amount of heat used to drive off residual hexane solvent (as done in Experiment 1), no attempt was made to duplicate the effects of the heat and pressure produced during screw pressing upon nutritive value. Further studies are needed upon the interactions between temperature, time, moisture content, pressure and CT upon the ileal digestibility of amino acids in CSM.

The value of dietary protein to monogastric animals is influenced largely by the proportion of the protein that can be digested and absorbed. The effect of heat treatment which leads to a reduction in protein digestion and amino acid absorption in the gut, has been extensively reviewed by Hurrell and Carpenter (1977) and Erbersdobler and Anderson (1983). In addition to the effects of sugar-protein Maillard reactions and isopeptide cross-links, it is well known that gossypol can also react with protein amino groups. As a number of amino acids such as lysine, threonine,

methionine and tryptophan are affected by heat treatment (Batterham 1992), it is possible that chemical reactions occur between amino acids within a protein molecule, in addition to the specific Maillard reaction between lysine and carbonyl groups of reducing sugars. As such, other amino acids in addition to those mentioned above may also be affected. In the present studies, heat treatment of CSM by autoclaving for 120 min significantly reduced the apparent ileal digestibility of all individual amino acids, except histidine. A finding of relevance to the present work is that of Moughan *et al.* (1995), who found that the apparent ileal digestibility of most amino acids for pigs fed a heated glucose/casein mixture was lower compared with an unheated mixture.

In the present study, whereas lysine was considerably less digestible than the other amino acids in the heated cottonseed materials and commercial CSM for both rats and pigs, this was not so for the rats fed the unheated cottonseed materials. It is known, that because of its free ε-amino group, lysine is the amino acid which is primarily affected when heat is applied to cottonseed protein (Erbersdobler and Anderson 1983). In addition the loss of available lysine in CSM is due partly to its reaction with the pigment gossypol present in cottonseed (Berardi and Goldblatt 1980), but other compounds are also involved (Martinez *et al.* 1967). A low apparent ileal threonine digestibility in heated materials was also found in this study. Batterham (1992) suggested that for lysine, threonine, methionine and tryptophan, heat induces changes which depress ileal digestibility slightly but result in a substantial proportion of these amino acids apparently being absorbed in inefficiently utilised forms. Thus, the conventional ileal digestibility assay may be unsuitable for fully assessing availability in heat-damaged meals.

The observed lower apparent ileal digestibility of protein and amino acids for pigs fed the CSM diets without PEG compared to those fed diets containing PEG found in the present studies can be attributed to the effects of dietary CT. PEG can specifically combine with CT to form CT-PEG complexes and the bonds in these complexes are stronger than the bonds in CT-protein complexes. Thus PEG can be used to displace protein from the CT-protein complexes without affecting the nutritional composition of the diet (Jones and Mangan 1977). Previous studies with PEG have shown that PEG addition *per se* had no intrinsic effect on protein digestibility in the absence of CT (Yu *et al.* 1995a, 1995b). In the present study, it was assumed that PEG completely bound with the CT released during digestion of the diets containing cottonseed hulls and CSM, and comparison between diets including or not including PEG allowed the effect of cottonseed CT to be defined.

The effects of CT in depressing apparent ileal protein digestibility may be explained either by a direct binding of CT to dietary proteins, by a reduced activity of

protein-degrading enzymes (Longstaff and McNab 1991; Jansman 1993), or by an increased secretion of endogenous proteins (Marquardt 1989; Jansman 1993). However, in recent work, no effect of cottonseed CT on endogenous ileal amino acid flow determined in the rat was found (Yu *et al.* 1995a). Commercial CSM produced in Australia contains between 150 to 300 g hulls kg^{-1} and has a CT content of 8 to 15 g kg^{-1} DM, 92% of which is bound to protein and fibre (Yu *et al.* 1993). Bound CT can react with protein in the monogastric digestive system (Yu *et al.* 1995b), although they appear to be relatively unreactive in the rumen (pH 7.0; Yu *et al.* 1995c). A possible explanation for the present result is that bound cottonseed CT were solubilised and released in the stomach of the pigs and were then available to react with proteins in the small intestine. The results obtained from rats in the present and previous studies (Yu *et al.* 1995b) are in line with the present result for pigs.

The third objective of this study was to determine whether the laboratory rat is an acceptable model animal for determining ileal amino acid digestibility in CSM for the growing pig. For an inter-species comparison of digestion to be valid, the species should be examined under physiologically comparable conditions. There appear to be major similarities between the rat and pig in digestive anatomy and physiology (Church and Pond 1988), requirements for nutrients (NRC 1978) and relative growth rates (Pullar and Webster 1977), especially when comparison is made at a physiologically comparable age (Donkoh *et al.* 1994). The body weights of both rats and pigs in this study were selected to correspond to the period of growth after weaning. The mean body weight of the rats (190 g) corresponded to 35% of mature body weight (NRC 1978) while that for the pigs corresponded to 12% of mature weight (Pond and Houpt 1978). Although the body weights, as proportions of mature weight were different, the rats (NRC 1962) and pigs (Headley *et al.* 1961) should have been in their linear phase of growth and so there should not have been any major differences in the stage of development of their digestive systems. Further the levels of food intake in the present experiments were chosen, so that relative food intake was comparable between the species.

Other studies (Taverner 1979; Picard *et al.* 1984; Smith *et al.* 1990; Donkoh *et al.* 1994) have shown similarities between rats and pigs for ileal amino acid digestibility in several protein sources. However, this may not be so for all feedstuffs and particularly for feedstuffs containing antinutritional factors (ANFs). Several studies indicate that rats and piglets may respond differently to ANFs in raw soyabean (Coms *et al.* 1967) and chickpeas (Visitpanich *et al.* 1985). Moreover, Moughan *et al.* (1984) found significant differences between rats and pigs for apparent ileal protein digestibility in peas. Huisman *et al.* (1989, 1991) compared the sensitivity of various animal species (pigs, rats, chickens and mice) to ANFs in legume seeds and

demonstrated that piglets were distinctly more sensitive to ANFs in beans and peas than rats and chickens and that results obtained with rats and chickens cannot be extrapolated to pigs. The findings of the present study indicate that there are significant differences in the apparent ileal digestibility of some amino acids in CSM between rats and pigs and particularly for the key amino acids, lysine and threonine. However, the pig was not distinctly more sensitive to cottonseed CT than the rat.

In summary, dietary cottonseed CT depressed apparent ileal protein digestibility for both the rat and the pig. Heat treatment (110°C for 2 h) did not diminish the reversible reactivity of CT with cottonseed protein in the small intestine of rats but rather appeared to increase it for threonine, tyrosine and lysine. There were differences in the apparent ileal digestibility of some amino acids in CSM between rats and pigs. Therefore, studies into the effects of CT should be carried out in the target animal species. Heat treatment significantly reduced apparent ileal amino acid digestibility in CSM, particularly for the essential amino acids lysine and threonine. In general, the CT effects in reducing apparent ileal amino acid digestibility of CSM were of smaller magnitude than those produced by heat treatment, but combinations of heat and CT were particularly detrimental in reducing apparent ileal digestibility of threonine, tyrosine and lysine.

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Chapter 9

GENERAL DISCUSSION

9. 1 INTRODUCTION

The nutritive value of feedstuffs depends on the contents of the various nutrients, their apparent digestibility and their utilisation after absorption. Both the digestibility and the utilisation of nutrients can be affected by the presence of antinutritional factors (ANFs), particularly for monogastric species. In the past decade research on ANFs, including condensed tannins (CT), in feedstuffs for both ruminant and monogastric animals has been stimulated by the search for alternative feedstuffs as protein sources. Cottonseed meal (CSM), as one of the alternatives, contains several ANFs, including gossypol, CT, phytate and other potential toxic factors (see Chapter 1).

The presence of ANFs, including gossypol and CT, is part of the host plant resistance mechanism in cotton for defence against attack by insects and pathogenic micro-organisms. Hence, from an agronomic point of view, their presence may be desirable. Katoh *et al.* (1989) found that the tannin content of leaves of Japanese cedar (*Cryptomeria japonica* D. Don) was reduced in areas with severe environmental pollution. This was accompanied by an increased predation of larvae of a herbivorous moth upon these leaves. This stresses the possible function of tannins in plants and their interaction with environmental factors.

CT in general may potentially exert a large number of antinutritional effects, but low concentrations (20-40 g kg^{-1} DM) in forages have been suggested to have beneficial effects for ruminants (Barry 1989). In this thesis, attention has been given to several aspects of this concept for the effects and mode of action of CT in CSM. They were studies in different animal species, including rats, pigs and sheep. Because rats have served as a model for many other monogastric species in nutritional studies, including those aiming at the elucidation of effects of dietary CT, most fundamental studies were performed with rats. Information on the nutritional effects of cottonseed CT in pigs is scarce. Therefore, one study was done with this species and the rat was evaluated as a model for the pig. Although several studies indicated that forages containing low levels of extractable CT have high nutritional value for ruminants, the effect of bound cottonseed CT on protein digestion in the ruminants has not been defined.

9. 2 CONDENSED TANNIN CONCENTRATION AND REACTIVITY

From results obtained in the studies reported in this thesis (Chapters 2, 7 and 8) it was concluded that CT are present in commercially produced CSM in significant concentrations. CT were present in the hulls (Table 9.1), but were not detected in the kernels. CT in cottonseed hulls, unlike CT in forages, were mainly in the protein- and

fibre-bound form. In general, only about 10-20% of total cottonseed CT were present in extractable forms, and hence their concentration would be greatly underestimated using conventional CT analytical methods which measure extractable CT only.

The results obtained from the studies in Chapters 7 and 8 showed that heat treating cottonseed hulls reduced the concentrations of total CT that could be extracted with acetone/water and SDS solution and determined with butanol/HCl. Application of heat treatment to the mixtures of kernel/hulls by autoclaving or in a forced-draught oven inactivated 29% and 13% of the total CT, respectively. Exposure to oxygen (including drying) can cause oxidative damage to CT (Goldstein and Swain 1965; McLeod 1974), and the above results may be explained by the oxidation of some CT to other compounds that may not be extracted and be detected by butanol/HCl.

Although the concentrations of total CT in cottonseed materials determined using the butanol/HCl method were reduced by the heat treatment, the results show that the effects of CT in reducing apparent ileal amino acid digestibility (Chapter 8) and in reducing N solubility and the rumen degradation of cottonseed proteins (Chapter 7) were not reduced by the heat applied, as judged by the responses to PEG. It seems that heat treatment did not diminish the reactivity of CT with cottonseed proteins. In fact, the increased responses to PEG in apparent ileal digestibility of threonine, methionine, tyrosine and lysine in heated compared to unheated CSM indicates that action of heat treatment may have increased the reactivity of CT with these amino acids, causing larger reductions in apparent ileal digestibility than heat or CT applied separately. These effects will be described further in Section 9. 3.

In both the monogastric and ruminant experiments, polyethylene glycol (MW 3,500) was either added to the diets (Chapters 5, 6 and 8), or the flasks used in the *in vitro* incubation (Chapters 3, 4 and 7) or continuously infused into the rumen (Chapter 3) to define the effect of CT. PEG has been shown to displace CT from CT-protein complexes (Jones and Mangan 1977) and completely binds extractable CT in forage at 1.8 mg mg^{-1} total CT (Barry and Forss 1983), preventing CT from binding to protein and releasing protein from CT-protein complexes in the rumen. The studies reported in this thesis demonstrated that 2.0 mg PEG mg^{-1} total CT was required to prevent or reverse the effect of CT in cottonseed hulls on N solubility and rumen degradation of the kernel proteins.

The lack of any PEG effect upon protein solubility and *in vitro* rumen degradability with pure cottonseed kernel, and upon ileal endogenous amino acid loss and ileal digestibility of N and amino acids in rats fed the diets containing no-hulls showed that PEG *per se* did not effect protein digestion and absorption for both

Table 9. 1 The concentrations of extractable and bound condensed tannin (g kg^{-1} DM) in the hulls of cottonseed cultivars, in cottonseed meal and other protein meals and in forages. All determinations used the butanol-HCl procedure.

	Condensed Tannin				
	Extract-able	Protein bound	Fibre bound	Total	Reference
Cottonseed cultivars (hulls only)					
Multiple host plant resistant					
MHR 10	7.0	26.8	7.0	40.8	This thesis (Chapter 2)
High gossypol					
HG 065	7.4	24.5	7.7	36.9	" "
High tannin					
HT-35-5-1	12.1	30.2	10.1	52.4	" "
HT-35-14-3	15.7	38.2	11.4	65.3	" "
Australian-bred cultivars					
Siokra L22	13.0	29.0	10.0	52.0	This thesis (Chapter 4)
Siokra 1-4	14.5	28.4	6.6	49.5	This thesis (Chapter 2)
Glandless					
DP 16	15.3	32.5	9.9	57.7	" "
Cottonseed meals (CSM)					
CSM	2.1	10.0	3.9	16.0	Terrill *et al.* (1992)
CSM Narrabri A	2.1	7.6	5.4	15.1	This thesis (Chapter 2)
CSM Narrabri B	1.8	5.2	2.1	9.1	This thesis (Chapter 7)
CSM Brisbane A	0.1	4.9	2.9	7.9	This thesis (Chapter 2)
CSM Brisbane B	0.6	3.1	2.0	5.7	This thesis (Chapter 7)
Heated and unheated mixtures of cottonseed kernel (CSK) and hulls					
Unheated CSK+					
16.7% hulls	2.4	4.6	0.9	7.9	This thesis (Chapter 8)
Heated CSK+16.7%					
heated hulls	0.8	3.9	0.9	5.6	" "
Unheated CSK+					
25% hulls	2.9	4.0	3.0	9.9	This thesis (Chapter 7)
Heated CSK+25%					
heated hulls	1.9	4.3	2.4	8.5	" "
Unheated CSK+					
50% hulls	5.0	7.0	5.3	17.3	This thesis (Chapter 7)
Heated CSK+50%					
heated hulls	6.0	6.1	3.1	15.2	" "
Protein meals					
Soya bean	1.0	0	0	1.0	Terrill *et al.* (1992)
Rapeseed	0.7	3.7	1.5	5.9	" "
Forage legumes					
Canary clover	83.0	54.0	6.0	143.0	" "
Birdsfoot trefoil	27.1	6.1	1.8	35.0	Wang *et al.* (1995)
Grasses					
Perennial ryegrass	1.1	0	0	1.1	Terrill *et al.* (1992)
Yorkshire fog	1.1	0.3	0.4	1.8	" "

ruminant and monogastric animals when the diet does not contain CT. Thus, the studies carried out in this thesis demonstrate that the application of PEG can be used to deduce nutritional effects of CT in cottonseed hulls, and mixtures of cottonseed kernel/hulls, as found for forages (Barry and Forss 1983; McNabb *et al.* 1993; Wang *et al.* 1995).

9. 3 NUTRITIONAL EFFECTS OF COTTONSEED CT IN MONOGASTRIC ANIMALS

Endogenous ileal amino acid loss was increased by inclusion of cottonseed hulls in an enzymically hydrolysed casein (EHC) based diets fed to growing rats (Chapter 5). This was probably caused by an effect of the hull fibre component on endogenous ileal amino acid loss, as the cottonseed CT did not appear to influence this loss.

Jansman (1993) fed pigs diets containing faba bean hulls with low and high concentrations of CT, and found that either dietary fibre and, or CT in faba bean led to a decreased true ileal digestibility of dietary protein and increased excretion of endogenously secreted proteins. The increase of endogenous excretion of protein may be due to an enhanced secretion of endogenous proteins or to a reduced degradation and reabsorption of endogenously secreted proteins. The latter could be relevant since Souffrant *et al.* (1986) found that 70 and 82% of endogenous secreted proteins in the alimentary tract of pigs are reabsorbed up to the terminal ileum and the rectum, respectively. Griffiths and Moseley (1980) suggested that dietary tannins may increase pancreatic secretion of digestive enzymes. Tannins may induce pancreatic secretion in a manner analogous to that of protease inhibitors from legume seeds (Liener 1989). The consumption of diets containing tannins was shown to specifically increase the size of the parotid glands in the rat and the synthesis and secretion of proline-rich proteins (PRPs; Mehansho *et al.* 1992). Tannin-induced PRPs were shown to have a very high binding affinity for tannins. The binding of tannins to both dietary and endogenous proteins has also been used to explain the reduced apparent digestibility of protein in tannin-containing diets. However, evidence for dietary cottonseed CT increasing endogenous ileal amino acid loss was not found in the study reported in this thesis.

It has been shown in Chapters 6 and 8 that inclusion of cottonseed hulls in the diets depressed apparent and true ileal digestibility of nitrogen and amino acids in rats, and reduced apparent ileal digestibility of nitrogen and amino acids in pigs (Table 9. 2). These depressions were mainly attributed to the cottonseed CT present in the diets. However, the response was different between protein sources in the diets. With the casein diets, all of the depression could be explained by the CT content of the hulls (Chapter 6), but with the cottonseed kernel diets, only part of the depression could be

explained by CT (Chapters 6 and 8). It seems that some unknown components of the hulls other than CT also depressed the apparent and true ileal digestibility of nitrogen and amino acids in cottonseed kernel. This was also shown again for chemically determined FDNB available lysine in Chapter 7. The responses to PEG in diets containing hulls reported in this thesis indicate that bound cottonseed CT were reacting with proteins in the monogastric digestive system. CT are known to be solubilised in the stomach (Jones and Mangan 1977), so that the most probable explanation for results obtained from Chapters 6 and 8 is that the bound CT in hulls were solubilised and released at the low pH in the stomach and were thus available to react with proteins in the small intestine.

The effects of CT in depressing apparent ileal protein digestibility may be explained either by a direct binding of CT to dietary proteins, by a reduced activity of protein-degrading enzymes (Longstaff and McNab 1991), or by increased secretion of endogenous proteins (digestive enzymes, mucus or mucosal cells; Mangan 1988; Marquardt 1989). Our results (Chapter 5) did not indicate that cottonseed CT increased endogenous protein. Therefore, the lowered apparent ileal digestibility of N and amino acids in the diets containing CT in cottonseed hulls may be attributed to a decrease in digestion and absorption as reflected by the true coefficients of digestibility of dietary protein.

It was also shown that cottonseed CT selectively bind to proteins *in vivo* (Chapters 6 and 8). There were differences in the effect of CT on apparent ileal digestibility of individual amino acids. The difference in apparent ileal amino acid digestibility between protein sources (casein and cottonseed kernel) suggests a difference in the intrinsic quality of the proteins. The major amino acid deficiency in CSM is that of lysine. For pigs, methionine, threonine, leucine and isoleucine become limiting after lysine (Chapter 1; Fisher and Quisenberry 1971). The ileal digestibility of lysine, threonine, isoleucine and methionine were more depressed by cottonseed CT compared with the other amino acids, particularly for heat treated or processed CSM. This may partly explain why these amino acids are deficient in commercially produced CSM. Differences in the effect of faba bean CT on ileal and faecal digestibility of individual amino acids have also been reported by Jansman (1993). It seems that CT may have a different affinity for proteins with different amino acid profiles.

The apparent ileal digestibility of lysine, methionine, threonine and isoleucine are much lower in commercially produced CSM than in unheated kernel diets that do not contain cottonseed hulls (Table 9. 2). The principle reason for this seems to be a combination of heat treatment and the presence of some cottonseed hulls in commercially produced CSM, with this combination causing major reductions in digestibility. As the

responses to PEG for these amino acids were greater in heated than in unheated CSM (Chapter 8), it seems that the action of autoclaving (110°C; 2 h) increased the reversible reactivity of cottonseed hull CT with kernel proteins. A further reason is the increase in endogenous ileal protein flow caused by the fibre component of hulls in the CSM.

Asquith and Butler (1986), in an *in vitro* study, noted that CT/protein interaction may be specific for different tannins as well as for different proteins. The high degree of interaction indicated that the differences in affinity were functionally significant. Hagerman and Butler (1981) found that sorghum tannins have a high affinity for proteins that are relatively large, with an open, loose structure and that are rich in hydrophobic amino acids, particularly proline. Cousins *et al.* (1981) in a study with sorghums containing different levels of CT showed that the apparent ileal digestibilities of tryptophan, histidine, glycine and proline were more depressed than for other amino acids in high-tannin varieties. Differences in protein structure and composition may account for the different responses to cottonseed CT observed in the studies reported in this thesis for cottonseed kernel and casein.

There appear to be major similarities between the rat and pig in digestive anatomy and physiology, requirements for nutrients and relative growth rates, especially when comparison is made at a physiologically comparable age (Donkoh *et al.* 1994). Other studies (Taverner 1979; Picard *et al.* 1984; Smith *et al.* 1990; Donkoh *et al.* 1994) have shown similarities between rats and pigs for ileal amino acid digestibility with several protein sources. However, this may not be so for all feedstuffs and particularly for feedstuffs containing antinutritional factors (ANFs). Several studies indicate that rats and piglets may respond differently to ANFs in raw soya bean (Coms *et al.* 1967) and chickpeas (Visitpanich *et al.* 1985). Moreover, Moughan *et al.* (1984) found significant differences between rats and pigs for apparent ileal protein digestibility in peas. Huisman *et al.* (1989, 1991) compared the sensitivity of various animal species (pigs, rats, chickens and mice) to ANFs in legume seeds and demonstrated that there is a difference in sensitivity between animal species. These authors concluded that piglets were distinctly more sensitive to ANFs in beans and peas than rats and chickens and that results obtained with rats and chickens cannot be extrapolated to pigs. The findings of this thesis indicate that there are significant differences in the apparent ileal digestibility of some amino acids in CSM between rats and pigs. Therefore, studies into the effects of CT should be carried out in the target animal species.

Table 9. 2 Apparent ileal digestibility of some essential amino acids in different animal species fed diets containing cottonseed hulls (CSH) or cottonseed meal (CSM)

		Apparent ileal digestibility of amino acids								
		Lysine		Methionine		Threonine		Isoleucine		
Diets	Species\PEG[1]	-	+	-	+	-	+	-	+	Reference
Casein diets[2]										
+0% CSH	rats	0.98	0.99	-	-	0.91	0.93	0.93	0.93	This thesis (Chapter 6)
+7% CSH	rats	0.95	0.98	-	-	0.89	0.92	0.88	0.91	" "
Unheated cottonseed kernel (CSK) diets										
+0% CSH	rats	0.82	0.80	0.84	0.84	0.74	0.73	0.81	0.80	This thesis (Chapter 6)
+4.6% CSH	rats	0.71	0.74	0.84	0.86	0.63	0.67	0.61	0.75	" "
Unheated/heated CSK diets[3]										
Unheated+5% CSH	rats	0.77	0.80	0.82[4]	0.84[4]	0.69	0.69	0.75	0.78	This thesis (Chapter 8)
Heated+5% CSH	rats	0.58	0.64	0.66[4]	0.74[4]	0.56	0.62	0.64	0.68	" "
Commercial CSM diets										
+40% CSM (Prepress solvent)	rats	0.50	0.52	0.61[4]	0.65[4]	0.50	0.53	0.57	0.62	" "
+40% CSM (Prepress solvent)	pigs	0.58	0.59	0.59[4]	0.59[4]	0.56	0.58	0.61	0.64	" "
+34% CSM (Direct solvent)	pigs	0.62	-	0.65	-	0.62	-	0.66	-	Tanksley *et al.* (1981)
+34% CSM (Screw press)	pigs	0.64	-	0.66	-	0.65	-	0.70	-	" "
+26% CSM (Prepress solvent)	pigs	0.56	-	0.72	-	0.51	-	0.63	-	Batterham *et al.* (1990)
+26% CSM (Prepress solvent)	pigs	0.67	-	0.80	-	0.64	-	0.73	-	" "

[1] Polyethylene glycol, MW 3,500; PEG-, without PEG addition; PEG+, with PEG addition.

[2] True ileal amino acid digestibility in rats fed casein based diets.

[3] Cottonseed kernel (CSK) and hulls (CSH) were heat-treated by autoclaving at 110°C for 120 min.

[4] Determined using conventional HCl amino acid hydrolysis. Data not included in the main body of the thesis (i.e. Chapter 8).

The value of dietary protein is influenced largely by the proportion of the protein that can be digested and absorbed. Application of heat treatment to proteins can reduce their digestion and absorption by the sugar-protein Maillard reaction and the formation of isopeptide cross-links (Hurrell and Carpenter 1977; Erbersdobler and Anderson 1983). As a number of amino acids such as lysine, threonine, methionine and tryptophan are affected by heat treatment (Batterham 1992), it is possible that chemical reactions occur between amino acids within a protein molecule, in addition to the specific Maillard reaction between lysine and carbonyl groups of reducing sugars. The results obtained from a study in Chapter 8 showed that heat treatment of cottonseed kernel/hulls by autoclaving at 110°C for 120 min significantly reduced the apparent ileal digestibility of all individual amino acids, particularly for lysine, threonine, isoleucine, and heat treatment may also cause other as yet unknown reaction involving CT that further lower apparent ileal digestibility of limiting essential amino acids. Lysine, tyrosine and cystine are more sensitive to heat treatment than are other amino acids (Ashes *et al.* 1984). A finding of relevance to this work is that of Moughan *et al.* (1995), who found that the apparent ileal digestibility of most amino acids for pigs fed a heated glucose/casein mixture was lower compared with an unheated mixture.

9. 4 NUTRITIONAL EFFECTS OF COTTONSEED CT IN RUMINANT ANIMALS

Condensed tannins in cottonseed hulls appear to reduce protein solubility in heated (Chapter 7) and unheated (Chapters 3 and 7) solvent extracted cottonseed kernels determined using both *in vitro* incubation in mineral buffer (pH 7.0) and the *in situ* polyester bag technique, and to reduce degradability of the two major seed storage proteins (52 and 48 kDa) present in heated (Chapter 7) and unheated (Chapters 4 and 7) cottonseed kernels estimated using *in vitro* incubations in rumen fluid and identification of individual proteins by SDS-PAGE. This is the result of the formation of stable complexes between CT and proteins, which do not dissociated at rumen pH (Jones and Mangan 1977).

A summary of the effects obtained upon N solubility is shown in Table 9. 3. Addition of hulls and application of heat consistently reduced N solubility, but the magnitude of the changes were small. PEG consistently increased N solubility measured *in vitro* but not *in situ.* Both the *in vitro* and *in situ* data indicate that a component in hulls other than CT also depressed solubility of kernel total nitrogen.

Table 9. 3 Effect of addition of hulls and heat treatment upon the nitrogen solubility of cottonseed kernel during *in vitro* incubation with mineral buffer[1] and *in situ* in the rumen[2]

	In vitro incubation Nitrogen Solubility (%)		*In situ* in the rumen N predicted Solubility (%)		References
PEG[3]	-	+	-	+	
Unheated cottonseed kernel					
+0% hulls	42	42	86	87	Chapter 3
+50 hulls	33	38	82	81	" "
+100% hulls	29	36	78	80	" "
+200% hulls	23	31	75	74	" "
Unheated/heated cottonseed kernel					
Unheated+0% hulls	49	50	ND[4]	ND	Chapter 7
Heated+0% hulls	45	47	ND	ND	" "
Unheated+50% hulls	37	46	ND	ND	" "
Heated+50% hulls	35	39	ND	ND	" "
Commercial cottonseed meal (CSM)					
CSM Narrabri	33	37	ND	ND	Chapter 7
CSM Brisbane	18	18	ND	ND	" "

[1] Samples were incubated with phosphate mineral buffer (pH 7.0) in a shaking water bath (90 rpm) at 39°C for 2 h.

[2] Samples suspended in the rumen of sheep using the polyester bag technique (Mehrez and Ørskov 1977).

[3] Polyethylene glycol (PEG), MW 3,500; PEG-, no PEG added to incubation; PEG+, PEG added to incubation.

[4] ND, not determined.

The data collected from *in vitro* rumen degradation studies in Chapters 4 and 7 have been used to calculate predicted rumen degradability, by correcting for rumen protein outflow rate using the equation: $P=A+[BC/(C+k)]$ (Ørskov and McDonald 1979). These data are shown in Table 9. 4, and represent predicted degradability of cottonseed proteins at a rumen outflow rate (k) of 0.046 h^{-1} (Ørskov and McDonald 1979), equivalent to a rumen retention time of 22 h. The values presented in Table 9. 4 are considered to be relative rather than absolute values, as degradation constants determined *in vitro* (A, B and C) may be different in magnitude to *in vivo*. Nevertheless, Table 9. 4 gives a good basis for comparing all the treatments applied in this thesis, as all the data were treated identically.

In general, predicted degradability values were higher for the cottonseed used in Chapter 4 than Chapter 7, perhaps representing differences between different sources of seed. However, in the absence of hull addition or heat, predicted degradability of

cottonseed kernel proteins was high at 76-94%. Adding hulls consistently lowered predicted degradability, with the effects being small in Chapter 4 but somewhat larger in Chapter 7. However, in Chapter 7 only part of the depression in predicted degradability could be explained by CT, as judged by the effects of PEG addition, emphasising that other components of hulls in addition to CT must also have been reducing degradability. Interestingly, as found for the monogastric data (Chapter 8), a combination of heat and CT produced the biggest reduction in rumen degradability (Chapter 7), with the responses to PEG showing that effects of CT were still reversible in heated meal. Heat-stimulated reaction with CT may change the properties of CSM proteins to decrease their solubility or the reaction sites for trypsin-like enzymes of ruminal microbes (Broderick and Craig 1980).

Table 9. 4 Effect of addition of hulls and heat treatment upon the predicted degradability[1] of cottonseed kernel proteins during *in vitro* incubation with rumen fluid

	52 kDa protein predicted degradability (%)		48 kDa protein predicted degradability (%)		References
PEG[2]	-	+	-	+	
Unheated cottonseed kernel					
+0% hulls	93	94	92	93	Chapter 4
+50 hulls	92	ND[3]	90	ND	" "
+100% hulls	86	92	85	92	" "
+200% hulls	76	ND	75	ND	" "
Unheated/heated cottonseed kernel					
Unheated+0% hulls	76	76	77	80	Chapter 7
Heated+0% hulls	68	66	71	66	" "
Unheated+50% hulls	61	68	65	68	" "
Heated+50% hulls	48	56	54	63	" "
Commercial cottonseed meal (CSM)					
CSM Narrabri	72	74	71	76	Chapter 7
CSM Brisbane	58	61	56	70	" "

[1] Predicted rumen protein degradability (P %) was calculated from the equation (Ørskov and McDonald 1979): $P=A+[BC/(C+k)]$ where A, B and C are constants, and k is the rumen protein outflow rate assumed to be 0.046 h^{-1} (Ørskov and McDonald 1979).

[2] Polyethylene glycol (PEG), MW 3,500; PEG-, no PEG added to incubation; PEG+, PEG added to incubation.

[3] ND, not determined.

Commercial CSM produced in Brisbane was of lower predicted rumen degradability than CSM produced at Narrabri, suggesting that greater heat had been

generated in processing at the Brisbane plant. The responses to PEG indicate that CT was contributing to reduce degradability in CSM manufactured at both locations.

Condensed tannins are able to bind simultaneously at multiple sites to protein by hydrogen bonding, and by hydrophobic and covalent interactions (Spencer *et al.* 1988b; Hagerman 1989). Presumably, CT-protein complexes are less susceptible to degradation by rumen micro-organisms. However, the hydrogen bonds are continuously broken and reformed randomly (McLeod 1974), while the degree of bonding between CT and protein is also affect by a large number of other factors, such as the chemical and molecular weight of the protein, presence of detergent, concentrations and nature of both CT and proteins (Asquith and Butler 1986; Horigome *et al.* 1988; Hagerman 1989). It is assumed that the optimum pH for the formation of insoluble CT-protein complexes is around the isoelectric point of the protein involved. The pH in the digestive tract of ruminants varies from 6-7 in the rumen to 2-3 in the abomasum. Therefore, the CT are able to complex with dietary proteins in the oral cavity during chewing and by-pass from rumen, and then may release protein in the abomasum or proximal duodenum at low pH for digestion and absorption. It also means CT, subsequently, may bind to other proteins of either exogenous (feed) or endogenous origin in the small intestine.

Rumen degradation of dietary protein is influenced by protein content, amino acid composition and protein structure (Romagnolo *et al.* 1990). The tertiary structure of protein affects the ability of the microbial population to gain access to peptide bonds. Ovalbumin, a soluble protein with a tight, convoluted tertiary structure (Cotta and Hespell 1986), is more slowly degraded in the rumen than casein (Mangan 1972), whilst proteins with numerous cross linkages are more resistant to degradation (Nugent and Mangan 1978). Mahadevan *et al.* (1980) concluded that the level of disulphide bonding within a protein was a major factor in determining its resistance to rumen degradation. As the sulphur amino acid content of cottonseed proteins is much lower than for ovalbumin, it seems that a low prevalence of disulphide bonds may be one of the factors contributing to the relatively high rumen degradability of globulin storage proteins in unheated cottonseed kernel.

The lack responses of cottonseed CT upon *in situ* N solubility may possibly be explained by CT being more effective at reducing protein degradation rather than protein solubility. Similar observation was also found by McNabb *et al.* (1995) for CT in the forage of *Lotus pedunculatus*. Spencer *et al.* (1988a) noted that the loss of N from synthetic-fibre bags suspended in the rumen measured the solubilization of plant protein, and that the rates of protein solubilization and degradation in the rumen were not necessarily similar. Positive correlation between predicted degradability of the two major kernel proteins (52 and 48 kDa) and solubility of total N *in vitro* was found

(Figure 9. 1). The data using in Figure 9. 1 were calculated from Tables 9. 3 and 9. 4. However, the correlation coefficient for 52 kDa protein was only r = 0.52 (p=0.08, n= 12), accounting for approximately 25% of the variation. The equation is:

$$Y = 49.97 + 0.41x \quad (1)$$

For the 48 kDa protein, the correlation coefficient was only r = 0.49 (p = 0.10, n = 12), accounting for approximately 25% of the variation. The equation is:

$$Y = 54.33 + 0.36x \quad (2)$$

For both equations 1 and 2, Y represents the predicted rumen degradability of the kernel proteins (52 or 48 kDa) and x represents the solubility of total N in buffer solution (pH 7.0).

This suggests that total N solubility *in vitro* was not a good index for estimating cottonseed protein degradation in the rumen. For estimating relative rumen degradation of cottonseed kernel proteins, the *in vitro* incubation with rumen fluid, followed by fractionation of individual proteins using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and their quantification using imaging densitometry, which has been developed and used in the studies reported in this thesis, is recommended.

Rumen DM digestibility of cottonseed hulls was 30-40% (Chapter 3; Tuncer *et al.* 1992). The DM digestibility was increased by PEG addition, indicating that bound CT was reducing the rumen digestion of cottonseed hulls. As cottonseed hulls are almost pure fibre, it seems that bound CT reduced fibre digestion. That CT in forages depressed fibre digestion in the rumen has been widely reported (Barry and Manley 1984; Barry *et al.* 1986; McAllister *et al.* 1993; Waghorn *et al.* 1994; Wang 1995). As Barry *et al.* (1986) and Wang (1995) showed the digestion of hemicellulose is more sensitive to forage CT than cellulose, it is possible that fibre digestion in the rumen begins to decline at a relative low level of CT. Therefore, Wang (1995) concluded that the optimum concentration of extractable CT in forages appears to be around 20 g kg^{-1} DM. At this level, CT had no effect on rumen fibre digestion, but substantially reduced protein degradation in the rumen and increased amino acid flux to the small intestine.

The overall results obtained from monogastric studies showed that cottonseed CT markedly depressed apparent ileal digestibility of amino acids. These effects occurred with the addition of low concentrations of hulls, and suggest that cottonseed hulls depressed digestion of cottonseed protein in the small intestine to a much greater extent than in the rumen, where large concentrations of hulls were required to produce depressions in degradability. Assuming the same effect occurs in the small intestine of the ruminant, bound CT in cottonseed products will probably have a small effect in reducing dietary protein degradation in the rumen, but may have a significant effect in

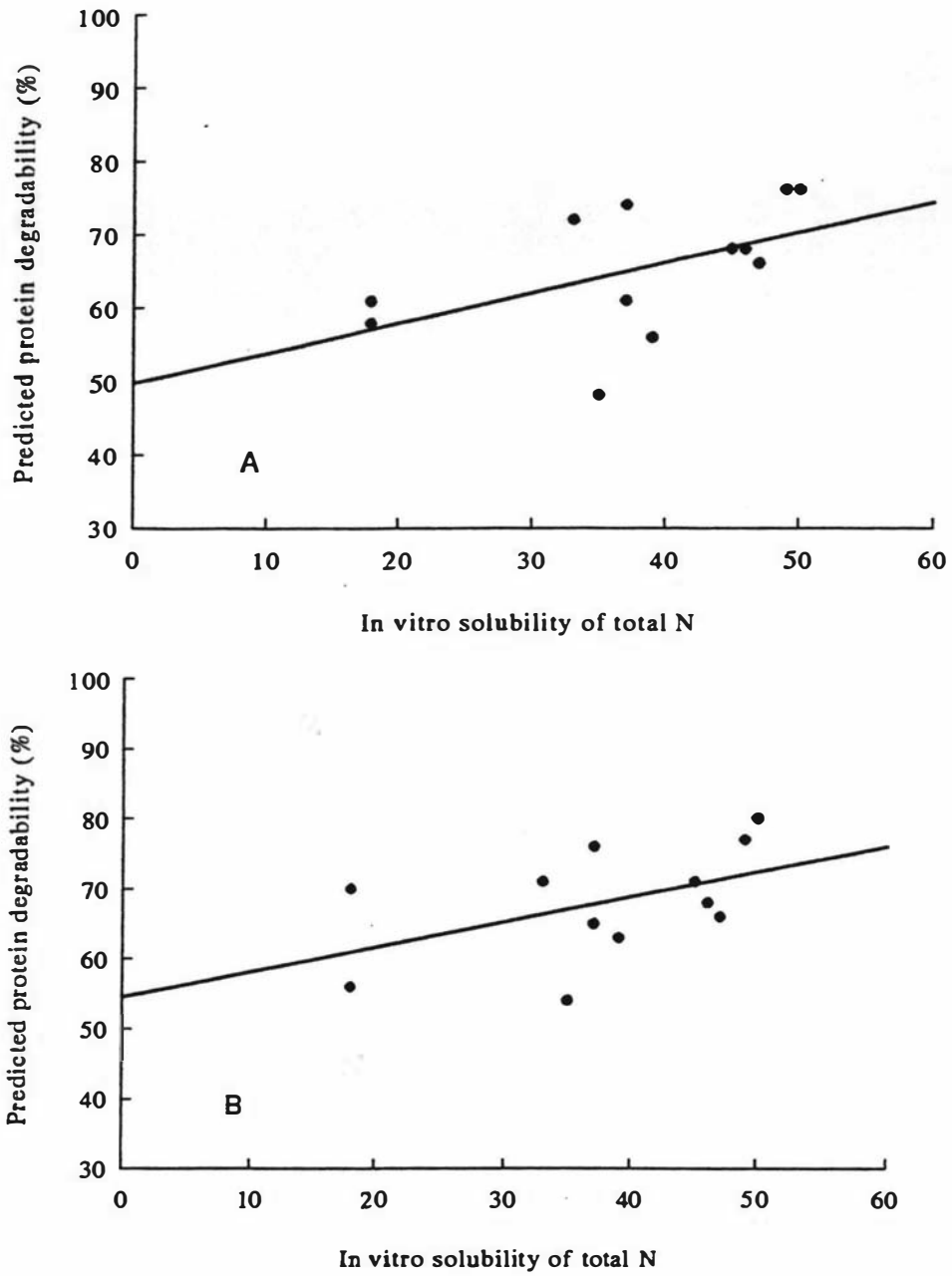

Fig 9. 1 The correlation between solubility of total N *in vitro* and predicted rumen degradability of the 52 kDa protein (A) and 48 kDa protein (B) in cottonseed kernel. For the 52-kDa protein, r = 0.52, n = 12, p = 0.08; For the 48 kDa protein, r = 0.49, n = 12, p = 0.10

reducing amino acid absorption from the small intestine. Thus, bound CT in cottonseed products, unlike extractable CT in forages, may have a greater adverse than beneficial effect upon dietary protein utilisation in ruminants. This may be due to bound CT being relatively unreactive and not moving from particle to particle at rumen pH, but being solubilised in the abomasum and being more reactive in the small intestine.

9.5 CONCLUSIONS

From the results generated in this thesis the following conclusions can be drawn:

1) Condensed tannins are present in commercially produced CSM in significant concentrations. CT are present in cottonseed hulls, but are not detected in the kernels. CT in cottonseed hulls, unlike CT in forages, are mainly in the protein- and fibre-bound form, with only 10-20% of total CT being extractable in acetone/water and determined with butanol/HCl. The presence of CT in commercially produced CSM is therefore due to the presence of some hulls in the CSM. During industrial processing, a component of hulls is left in the CSM to allow more efficient extraction of the oil (the most valuable commodity) during screw pressing.

2) Normal analytical methodology measures extractable CT only, and this grossly underestimates the total concentration of CT in CSM. For use with CSM and other processed meals, analytical methods are required which measure bound as well extractable CT.

3) Ileal amino acid digestibility is high in monogastric animals fed unheated solvent extracted cottonseed kernel diets, where no hull is present. Relative to unheated solvent extracted kernels, commercially produced CSM is of low protein nutritional value for monogastric animals. In particular, the ileal digestibility of lysine, methionine and threonine are low. The low ileal amino acid digestibility in CSM is probably due to a combination of heat applied during processing and the presence of some hulls in the CSM, with approximately half the hulls effect being explained by their CT content. It seems that bound CT in cottonseed hulls are solubilised in the monogastric stomach and then reduce amino acid digestion in the small intestine. Leaving some hulls in CSM substantially lowers the efficiency with which amino acids can be digested and absorbed from the small intestine in monogastric animals. The study has shown that cottonseed hulls are not an inert substance for monogastric animals; rather they are antinutritional and reduce the efficiency of protein digestion.

The study has also shown that the large amount of heat applied during normal commercial CSM processing reduces the digestion of amino acids by monogastric animals. A combination of heat and CT from hulls is damaging for lowering amino acid digestion, particularly for the dietary limiting essential amino acids lysine, methionine and threonine. Where CSM is intended to be used in the monogastric livestock industries, it is recommended that the level of hulls in CSM should be reduced to the lowest possible levels and that the amount of heat applied should be the minimum amount to bind gossypol.

4) In the absence of hulls or heat, rumen degradation of cottonseed kernel proteins is high (76-94%). As CT in forage plants substantially reduces rumen protein degradation in ruminants, an objective of the project was to study the effect of CT in CSM upon rumen protein solubility and degradation. Although action of bound cottonseed CT did reduce the degradation of cottonseed kernel proteins by rumen micro-organisms, the effect seems of small magnitude. The effect is smaller than that produced by similar concentrations of forage CT, and is probably explained by bound CT being relatively unreactive at rumen pH (6.0-7.0). Total N solubility measured either *in vitro* or *in situ* was not a good index for estimating cottonseed protein degradation in the rumen. For estimating rumen degradation of cottonseed proteins, the *in vitro* incubation with rumen fluid, followed by fractionation of individual proteins using SDS-PAGE and their quantification using imaging densitometry should be used.

Whilst the presence of CT will give some protection of cottonseed kernel proteins against rumen fermentation, the effects seem small in unheated CSM but tended to be somewhat greater in the presence of heat. Further research is necessary to accurately define the amount of heat (temperature, time) required to give optimum rumen by-pass of CSM proteins. As the moisture and pressure used in CSM processing enhances the heating process, making it more effective than dry heat at a given temperature, moisture content and pressure should therefore be included as factors in future heating studies. Interactions among heating temperature, heating time, moisture content, hulls and CT need to be defined in future work.

As cottonseed CT lowered the protein and amino acid digestion in the small intestine of monogastric animals, it is likely that this will also happen in ruminants. The possibility therefore exists that with ruminants, negative effects of cottonseed CT in the small intestine may outweigh any beneficial effects in reducing rumen degradation of kernel proteins.

5) Action of cottonseed CT lowered the digestion of cottonseed hulls by rumen micro-organisms. As the hulls are almost pure fibre, it seems that bound CT may reduce rumen fibre digestion.

6) From the results of the thesis it seems that CSM intended for the monogastric industries should be processed differently from CSM intended for the ruminant industries. The effects of CT and their continued reactively after heating have been accurately defined in this thesis. Future work is necessary to accurately define the extent of heating required to produce CSM of optimum nutritional value for ruminant and monogastric livestock, and the interactions with heat, CT and moisture content and their effect upon protein nutritional value also need to be defined. It is recognised that some heating is necessary, to soften the seed and to bind gossypol, but the amount of heat required is likely to differ substantially in CSM intended for ruminant and monogastric markets.

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