Chapter 2
Materials and Methods

2.1 Introduction

The study consisted of three sections. The first was a digestibility trial involving feed intake, faeces and urine excretion and certain rumen parameters being measured. The second was an *in sacco* trial to measure the DM and NDF degradability of the feeds, and the third was a rumen evacuation trial measuring the passage rate of the feeds.
All three sections to the trail were conducted at the same location and involved the same animals. Measurements and analytical methods will be discussed under each section concerned.

The aim of this study was to determine the nutritive value of the two leguminous forage shrubs, namely *C. sturtii* and *S. microphylla,* compared to *M. sativa* (SA Standard cultivar), which is a well-known leguminous forage plant. By comparing the two shrubs one can determine the value to the animals and whether or not it will be sustainable to utilize these shrubs as fodder for sheep in dry areas.

2.2. Location

The trial was conducted on the Hatfield Experimental Farm of the University of Pretoria, South Africa (co-ordinates 025°15'28.9"E, 25°45'03.6"S). Pretoria is situated in the Gauteng Province at an altitude of 1360m. It is a summer rainfall area with an average annual rainfall of 650mm, of which half occurs during November to January. The temperatures are moderate in winter and occasional frost does occur. The average daily minimum and maximum temperatures are 10.3 and 24.5ºC respectively.
The soil type is a Hutton form (MacVicar *et al.*, 1977), is well-drained and slightly acidic and has a good nutrient status. The Hutton type is a deep-clay-loam soil with approximately 25% clay and an effective depth of 600mm+.

2.3. Material

The *C. sturtii* (Origin: Australia), *S. microphylla* (Origin: South Africa) and *M. sativa* (SA Standard cultivar) were planted and harvested on the Hatfield Experimental farm. The *Cassia* plants were established from seedlings in February 2002 and were approximately 3 years old when harvested, while the *Sutherlandia* was seeded *in situ* in the spring/summer of the 2003/2004 season. Both plants were established under dry land conditions. The *M. sativa* was cultivated during late summer 2003 under dry land conditions. The whole plants were harvested from May to June 2004 at a height of 30cm from ground level and then sun-dried. To minimize selection of the material all harvested material was milled through a 1cm sieve size. The physical appearance of *C. sturtii* and *S. microphylla* before harvesting, are presented in Figure 2.1 and 2.2.

Figure 2.1 Physical appearance of *C. sturtii* before harvesting on the Hatfield Experimental Farm

Figure 2.2 Physical appearance of *S. microphylla* before harvesting on the Hatfield Experimental Farm

2.4. Animals

Six adult cannulated Döhne Merino wethers were used. Three of the sheep were fitted with small rumen cannulae (5cm diameter) and the other three with large rumen cannulae (10cm diameter). The same animals were used during each section of the trial.
A randomised block design was used to allocate animals to treatments. Each sheep received all three treatments, starting with one and then moving to the next treatment in the following period. The three treatments included feeding with *Cassia sturtii, Sutherlandia microphylla* or *Medicago sativa.* The animals were kept in individual cages in the sheep metabolism house of the Small Stock Section on the Hatfield Experimental Farm for the full duration of the experiment. For the digestibility trial they were housed on concrete floors in larger cages during the adaptation period and were put into the metabolic crates three days before the experimental period and remained in the crates for the duration of the experimental period. Thereafter they were once again placed in larger cages on concrete floors until the next experimental period. During the other two trial sections, the sheep remained on the concrete floors and sampled as necessary.

All animals received the necessary vaccinations (pulpy kidney and blue tongue) and were dosed for internal parasites before the start of the experiment. Animals were fed twice a day and

received fresh water on an *ad-lib* basis. The metabolic house was cleaned on a daily basis. The ethics committee (project nr. AUCC 041215-031) of the University of Pretoria, Animal and Wildlife Sciences Department evaluated the trial before any research was undertaken and approved the procedures.

2.5. Digestibility trial

Voluntary intake and digestibility was determined by offering animals a known quantity of feed *ad lib* and determining the amount remaining at the end of the feeding period. Digestion and retention coefficients were determined by collecting all the excreta (mainly urine and faeces) and analysing feed and excreta samples.

The digestibility trial was conducted using all six cannulated animals. The trial consisted of three periods with each animal allocated to a treatment during the period (randomized block design – see Table 2.1). This allowed for a more accurate statistical analysis to minimize the error.
In Table 2.1 the experimental outline of the digestibility trial is presented.

Table 2.1 Experimental outline of the digestibility trial conducted on six cannulated animals using the three forages *S. microphylla, C. sturtii* and *M. sativa*

Period 1 (16 days)	*S. microphylla* (2 sheep)	*C. sturtii* (2 sheep)	*M. sativa* (2 sheep)
Period 2 (16 days)	*C. sturtii* *(2 sheep)*	*M. sativa* (2 sheep)	*S. microphylla* (2 sheep)
Period 3 (16 days)	*M. sativa* (2 sheep)	*S. microphylla* *(2 sheep)*	*C. sturtii* (2 sheep)

The experimental procedure lasted for approximately 20 days. The first 14 days consisted of an adaptation period for the experimental diets, while the following 6 days were used for data collection, including collection of feed, orts, faeces and urine. The animals were fed *ad lib* dry feed per day during the adaptation and experimental period.

2.6 Measurements and preparation for chemical analyses

Animals were weighed at the start and end of each period to determine the change in body weight during the trial. Weighing was after a period of overnight starvation (food and water).
Individual intake was recorded by weighing the feed offered and the orts daily. A daily sample of each feed (as fed) and feed refusals was obtained and stored for later analysis. At the end of each period the samples from each feed were thoroughly mixed together, oven dried at 55 ºC for 48 hours and then stored until analyzed. Each animal was equipped with a faecal bag to record daily faecal excretion. Faeces were collected twice a day, weighed and a 10% representative sample was taken. The samples were pooled and then dried for 48 hours at 100ºC in a force draught oven, milled and stored for later analysis. The metabolic crates used were such that the total urine output could be collected and measured daily. This was collected in a container that contained 25ml of a 10% H_2SO_4 solution to prevent loss of urinary ammonia. The urine plus acid was then stored in plastic vials and frozen until analyzed for allantoin and uric acid.

Three random samples of each feed were taken at the start of each period, then pooled into one sample to determine the chemical composition of the feeds. DM, ash, NDF and ADF, ADL, CF, CP and minerals were determined.
Feed samples (5 – 10%) were collected at each feeding and all faecal and urine excretions collected were stored at –10ºC until the end of the collection period. At the end of the collection period, total faeces and urine collected was mixed and sub-sampled. The urine was analysed for protein/nitrogen concentration. Feed and faecal samples were ground to pass through a 1mm sieve and dried to determine DM concentration according to the AOAC 934.01 (2000). Total nitrogen was determined using the Dumas/Leco technique described in the AOAC 968.06 (2000). NDF and ADF was determined as described by Goering and Van Soest (1970).

On days 3 and 4 during the experimental period samples of rumen fluid were taken over a period, giving 12 samples; 08:00, 12:00, 16:00, 20:00, 24:00, 04:00, 10:00, 14:00, 18:00, 22:00, 02:00 and 06:00 respectively.
Rumen contents were collected with the aid of a 60ml syringe connected to a plastic tube. Contents were drawn up from a few locations in the rumen into the tube by aid of suction caused by the syringe. Approximately 150ml of rumen contents was collected per animal during each sampling period.

The rumen contents were drained through cheesecloth, with the solids being discarded. A 30ml sample of rumen fluid was put into a container with 5ml 0.5 M H_2SO_4 to be used for the determination of rumen ammonia nitrogen (NH_3-N) concentration with an auto analyzer (Broderick & Kang, 1980). A further 10ml sample was placed in a container with 1.0ml of a 10% sodium hydroxide (NaOH) solution for VFA analysis, using the gas chromatographic method (Webb, 1994). The samples were then pooled for each animal and then frozen for later analysis.

2.7 Chemical analysis

2.7.1 Dry matter and Ash

Dry matter was determined on the feed, orts, faeces, *in sacco* and rumen content samples. (AOAC: 934.01, 2000).

%DM = (Mass of oven dry sample/Mass of air dry sample) x 100

%Ash = (Mass of Ash/Mass of air dry sample) x 100

%OM = 100 - %Ash (DM-basis)

2.7.2 Nitrogen and crude protein

CP was determined on the feed, orts and faeces samples. It was determined using the Dumas method described in the AOAC 968.06 (2000).

2.7.3 Crude fibre

CF was determined for the three feed samples using the ceramic fibre filter method described by the AOAC 962.09 (2000).

Crude fibre % = [(W2 – W3)-(B2 – B3)]/W1 x 100

Where W1 = original sample weight
W2 = sample weight after drying at 110ºC
W3 = sample weight after ashing at 550ºC
Where B2 and B3 are the average weights of all the blanks after drying and ashing respectively.

2.7.4 Neutral detergent fibre (NDF)

NDF was determined for the feed, orts, faeces, rumen content and *in sacco* samples. The analysis was done using the Dosi fibre system (Robertson & Van Soest, 1981).

NDF was calculated as follows:

%NDF = [(dry mass of NDS extracted sample (g) – mass of ash (g))/sample mass (g)] x 100

2.7.5 Acid detergent fibre (ADF)

ADF was determined according to the method by Goering & Van Soest (1988).

ADF was calculated as follows:

% ADF = [(dry mass of sample after ADS extraction (g) – mass of ash (g))/sample mass (g) x 100

2.7.6 Acid detergent lignin (ADL)

ADL was determined according to the method by Goering & Van Soest (1988).

ADL was calculated as follows:

%ADL = [(W1 – W2)/W0 (sample mass)] x 100

Where W0 = original sample mass
W1 = weight after acid extraction and first overnight drying
W2 = weight after ashing at 550ºC for three hours

2.7.7 Minerals

Samples for mineral analysis (Ca, Mg, Zn, Cu, Mn, Fe) were prepared by the method of the AOAC 935.13 (2000). Sample preparation for phosphorus was done using the AOAC 968.08 (2000) method.
Atomic absorption spectrophotometery was used to determine calcium (Ca), magnesium (Mg), zinc (Zn), copper (Cu), manganese (Mn) and iron (Fe) concentrations according to the method by Giron (1973). P concentration was determined using the method by AOAC 965.17 (2000).

The mineral concentration was estimated as follows:

% Element = (µg/g of element in sample solution x df x 0.0001)/sample weight (g)
For Ca, Mg and P which is expressed as g/kg.

mg/kg Element = (µg/g of element in sample solution x df)/sample weight (g)
For Fe, Zn, Cu and Mn which is expressed a parts per million (mg/kg)

Where df = dilution factor

2.7.8 Rumen NH_3-N

The rumen fluid samples were analyzed for rumen ammonia nitrogen concentration, using a Technicon Auto-Analyzer (Broderick & Kang, 1980).

2.7.9 Rumen Volatile fatty acids (VFA)

The determination of VFA's in the rumen is done by using the Gas chromatographic method. The apparatus used was a Varian 3300 FID Detector Gas Chromatograph, of which the gas is hydrogen and air (Webb, 1994).

The results were calculated as follows:

mg/100ml VFA in sample = Peak area of sample/ Peak area of standard x standard concentration x dilution factors

The above result can then be divided by the molecular mass of the VFA to obtain concentration in mmol/100ml sample (Webb, 1994).

2.8 Estimation of microbial protein supply using urinary allantoin

The urine was analyzed for allantoin to determine the microbial protein concentration in the method described by Pentz (1969).

2.8.1 Sample preparation

1) Total urine excretion was collected in a plastic container on a 24 hour basis from the animal for 5 – 7 days.
2) 100ml of a 10% H_2SO_4 was added to the urine to ensure the pH remained less than 3.
3) Each morning the quantity of urine voided was measured, and 3 – 5l of water was added to prevent precipitation of uric acid.
4) A subsample (20 – 50ml) of diluted urine was taken, and frozen for allantoin analysis.

5) Urine was then diluted in the laboratory as follows; for allantoin determination dilution rate was 1 in 30 times (1ml of urine + 29 ml distilled water)

2.8.2 Allantoin

A sample of urine (1 – 2ml) was transferred to a 50ml volumetric flask and diluted to volume with distilled water. 5ml of the diluted urine was pipetted into a Pyrex tube graduated at 25ml and 1ml of a 0.5 M NaOH was added and vigorously boiled in a water bath for 7 minutes and then, on removal, immersed in a water bath at 20ºC. 1ml of 0.5 M NaOH was added with 5 drops additional to adjust acidity to approximately 0.02M. Five ml of the standard solution of allantoate are pipetted into a separate tube and 5 drops of 0.5 M HCl added. Then 1ml of the phenylhydrazine solution was added to each tube. The tubes were shaken and placed in a boiling water bath for 2 minutes then immediately plunged into an ice-salt bath at -10ºC and chilled for 3 minutes. On removal, 3ml of a chilled hydrochloric acid was added to each tube and 1ml of potassium ferricyanide solution. The contents were well mixed. After 30 minutes the tubes were filled to the mark with distilled water and compared in a Duboscq colorimeter (Pentz, 1969).

The calculation is as follows:

S/U x 0.1 x 0.738 x 50/5 x 100/X = S/U x 73.8/X = mg allantoin in 100ml urine (Pentz, 1969)

Where: S = reading of the standard
U = reading of the unknown
X = number of ml of urine used for initial dilution to 50ml
0.738 = dilution factor for conversion of allantoate to allantoin

2.8.3 Microbial Nitrogen supply and uptake

Microbial protein supply was estimated according to the equation proposed by Puchala and Kulasek (1992)

$$Y = e^{(0.830 + 2.089x)}$$

Where: Y = microbial nitrogen supplied (g/day)
X = urinary excretion of allantoin (mg/day)

The efficiency of the microbial protein supply in the rumen is expressed as microbial nitrogen supply (g/day) per kilogram digestible organic matter intake (DOMI) (g DM/day/kg $W^{0.75}$).

2.9. Rumen degradability

Ruminal dry matter and neutral detergent fibre digestibility was determined using nylon bags.
Three animals with large rumen fistula were used to measure *In situ* rumen DM and NDF degradability of the three fodder species using the technique described by Osuji *et al.* (1993a).
The animals were adapted to the feed for three days before incubation started.
The sheep were fed twice daily during the 15 day experimental period with 3 days to assess each plant.
The dried feed samples (determined dry matter concentration) were milled through a 2mm screen.
Bags (140 x 90mm) made of polyester cloth, with an average pore size of 53µm were used. The bags were dried at 60 ºC for 30 minutes and then weighed.
Approximately 5g of dry sample was weighed into the oven dried bags in duplicate, which were then tied off with 100% polyester string. The bags were attached to a stainless steel disc (140g, 45mm diameter, 11mm thick and with ten holes around the edge) using the polyester string. A 50cm nylon string was attached to the metal disc to anchor the bags deep inside the rumen.
A 3 x 3 factorial experimental layout was used as illustrated in Table 2.2.

Table 2.2 Experimental layout of the rumen degradability trial

Treatment	Period 1	Period 2	Period 3
Lucerne	Animal 1	Animal 3	Animal 2
Sutherlandia	Animal 2	Animal 1	Animal 3
Cassia	Animal 3	Animal 2	Animal 1

The bags were all incubated in the rumen at once on day 1 and removed one by one using the sequential withdrawal method (Osuji *et al.,* 1993a). Bags were withdrawn at 0, 4, 8, 16, 24, 48, 72 hours respectively. After each incubation time, one bag was removed per animal, immediately dipped in ice water to prevent further microbial activity. Each bag was then rinsed under running cold water to remove microbes and degraded material smaller than the bag pores. The bags were then frozen until all bags had been removed and washed.

All the bags were then dried in a force draught oven at 60ºC for 48 hours. The bags, with their contents, were then weighed. The procedure was repeated twice to give six replications for each treatment per animal treatment (Osuji *et al.,* 1993a).

The dry matter and NDF of the residue samples were then determined using the same analysis as with feed samples.

The disappearance was calculated using the following formula (Osuji *et al.,* 1993a):

Disappearence = (SWa – BW) x DMa – (SWb – BW) x DMb)/ (SWa – BW) x DMa

Where: SWa = weight of original sample + nylon bag

BW = weight of empty nylon bag

SWb = weight of the sample + nylon bad after incubation

DMa = Dry matter/NDF of feed sample

DMb = Dry matter/NDF of residue sample

These data were then processed electronically and plotted against time using the model of DM disappearance proposed by Orskov & McDonald (1979) which summarizes the data and derives degradation parameters.

$$Y = a + b\,(1 - e^{-ct})$$

Where: Y = degradability at time (t)

a = intercept

b= potentially degradable fraction

c = rate of degradation of b

2.10. Rumen kinetics

The total weight of rumen contents and the passage rate were estimated by manually emptying the rumen of each animal at different times. There should be a minimum of 24 hours between consecutive emptyings (Robinson *et al.*, 1987; Tamminga *et al.*, 1989; Osuji *et al.*, 1993a). Figure 2.3 shows how the emptying was undertaken during the trial

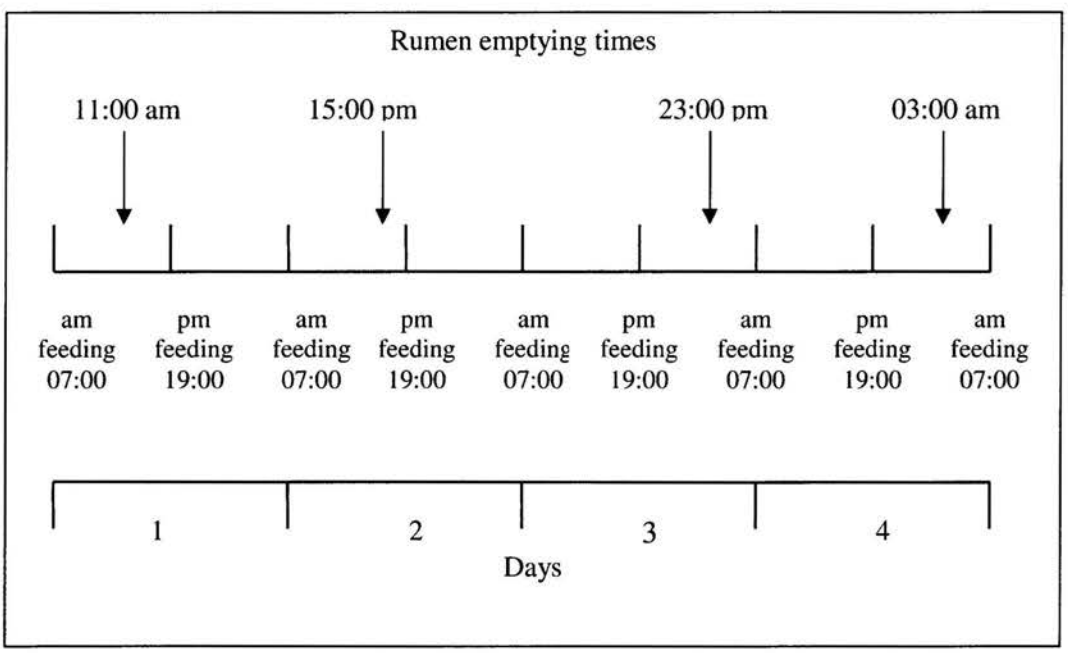

Figure 2.3 Rumen evacuation time schedule (4 & 8 hours after feeding)

Procedure used for estimating rumen volume by evacuation (Osuji *et al.,* 1993a)

1. Remove the cover of the rumen cannula and empty all rumen contents by hand into a barrel, keeping the barrel in warm water.
2. Weigh all the material, mix thoroughly and take a 2.0 – 2.5 kg sample
3. Return the remaining material to the rumen as soon as possible (The procedure should not exceed 10 minutes and the rumen should only be empty for 2-3 minutes.)
4. Dry rumen samples at 100℃ for 24 hours to determine dry matter content of rumen digesta
5. Dry samples of rumen content for analysis at 60°C for 48 hours.

The kinetics of rumen NDF intake, passage rate and digestion can be calculated using the model described by Robinson *et al.* (1987), assuming steady state conditions in the rumen.

Rate of intake (ki per hour) = 1/24 x (intake, kg/day)/ (rumen pool size, kg)
Rate of passage (kp per hour) = 1/24 x (faecal flow, kg/day)/ (rumen pool size, kg)
Rate of digestion (kd per hour) = ki – kp

Where: Intake = kg NDF (DM basis) per day
Pool size = kg NDF (DM basis) in the rumen
Faecal flow = kg NDF (DM basis) excreted per day (Robinson *et al.,* 1987)

The extent of digestion of a feed is controlled by the relationship between rate of digestion and rate of passage (Mertens, 1993), therefore:

% NDF digested in rumen = kd/(kp+kd)
% NDF passing from rumen = kp/(kp+kd)

2.11. Statistical analysis of data

An analysis of variance with the ANOVA model (Statistical Analysis Systems, 2005) was used to determine the significant difference between different fodder species/treatments, period and animal effects for the balanced data. Means and standard errors (se) were calculated.
Significance of difference (5%) between means was determined by using Fischer's test (Samuels, 1989).