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# The determination of digestibility of *Atriplex nummularia* cv. De Kock with different techniques


**by**

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Submitted in partial fulfilment of the requirements for the degree

**M.Sc. (Agric)(Animal Nutrition)**

in the


Department of Animal and Wildlife Sciences
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Pretoria

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# DECLARATION

I declare that this dissertation, for the degree M.Sc. (Agric) at the University of Pretoria, has not been submitted by me for a degree at any other university.

A. van der Baan

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# ABSTRACT

**The determination of digestibility of *Atriplex nummularia* cv. De Kock with different techniques**

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The aim of the study was to determine the digestibility of *Atriplex nummularia* cv. De Kock, supplemented with three levels (15%, 30%, and 45%) of either maize or barley, using different *in vitro* techniques. An *in vivo* digestibility trial was conducted, together with a number of *in vitro* trials.

An important development has been the introduction of biological methods (Jones & Theodorou, 2000). Three digestion techniques that simulate the digestion process are currently available to determine the nutritive value of ruminant feeds:

1. Digestion with rumen micro-organisms as in the work of Tilley & Terry (1963) or gas method (Menke *et al.,* 1979). Digestion with faeces micro-organisms (El Shaer *et al*., 1987), as an alternative to rumen fluid inoculum.
2. Cellulase methods

3. *In situ* incubations of samples in nylon bags in the rumen.

The results of this study showed no significant difference (P>0.05) between the rumen- and faeces inoculum *in vitro* techniques, but they did differ significantly from the gas production and cellulase techniques. There was also no significant difference between the gas production and cellulase techniques. Organic matter digestibility (OMD %) of the *in vitro* techniques differed significantly from the *in vivo* OMD % values.
There are several possible explanations for the difference between the *in vivo* and *in vitro* OMD %. 1. Practical mistakes could have been made. 2. The simulation of the rumen motility *in vitro* is often difficult and it may be that all the feed particles did not have the same exposure to the micro-organisms, as it would have in the rumen of an animal. The different rumen pools are also not fully represented *in vitro*. 3. The fermentation characteristics and microbial constitution of the rumen inocula differ, between the animal used for the *in vivo* digestibility trial and the animals used for rumen inocula collection. 4. With *in vivo* digestibility the time of digestion is not known, and therefore the time of rumen and gastric digestion *in vitro* could have been too long or too short.

It was found that the *in vitro* faeces technique of El Shaer *et al*., (1987) is an easier and cheaper alternative to the classic rumen fluid *in vitro* technique of Tilley & Terry (1963), as modified by Engels & Van der Merwe (1967). The *in vitro* faeces technique uses faeces as an inoculum and therefore solves the problems associated with the use of cannulated animals. The gas production *in vitro* technique has certain advantages, but still has the disadvantage of needing cannulated animals for rumen inocula. The cellulase-based i*n vitro* technique in contrast eliminates the use of cannulated animals. Although the *in vitro* gas production technique of Pienaar (1994) and the cellulase *in vitro* technique could both be used to determine the OMD % of *Atriplex nummularia* cv. De Kock, the values will be lower than *in vivo* determinations.

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# UITTREKSEL

**Die bepaling van die verteerbaarheid van *Atriplex nummularia* cv. De Kock deur gebruik te maak van verskillende tegnieke**

**deur**

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Die doel van die studie was om te bepaal wat die verteerbaarheid van *Atriplex nummularia* cv. De Kock, gesupplementeer met drie vlakke (15%, 30%, en 45%) van mielies of gars sal wees, deur verskillende laboratorium tegnieke te gebruik. ‘n *In vivo* verteringsproef is saam met verskeie *in vitro* proewe gedoen.

‘n Belangrike ontwikkeling was die bekendstelling van biologiese metodes (Jones & Theodorou, 2000). Drie verteringstegnieke wat die verteringsproses simuleer is huidiglik beskikbaar om die voedingswaarde van herkouervoere te bepaal:

1. Vertering met rumen mikrobes soos in die studie van Tilley & Terry (1963) of die gasproduksie metode (Menke *et al.*, 1979). Vertering met mikrobes

vanaf mis, word as alternatief vir rumenvloeistof inokulum gebruik (El Shaer *et al*., 1987).

2. Sellulase metodes
3. *In situ* inkubasies van monsters in nylon sakkies in die rumen.

Die resultate van hierdie studie het geen betekenisvolle verskille ($p>0.05$) tussen die rumen- en mis inokulum *in vitro* metodes getoon nie, maar het van die gasproduksie en sellulase metodes verskil. Daar was ook geen betekenisvolle verskille tussen die gasproduksie en sellulase metodes nie. Organiese materiaal verteerbaarheid % (OMV %) van die *in vitro* metodes het betekenisvol van die *in vivo* OMV % waardes verskil.

Daar is veskeie moontlikhede wat vir die verskille tussen die *in vitro* en *in vivo* OMV % verantwoordelik kon wees. 1. Praktiese foute kan gemaak word. 2. Die simulasie van die rumen motiliteit *in vitro* is nie maklik nie en al die voerpartikels het nie dieselfde blootstelling aan die mikrobe aktiwiteit, soos in die rumen gehad nie. 3. Die verskillende rumenpoele word nie ten volle verteenwoordig *in vitro* nie. 4. Die verteringseienskappe van die rumen inokulum verskil, tussen die diere gebruik vir die *in vivo* verteringsproef en die diere gebruik vir die kolleksie van rumen inokulum. 5. Met die *in vivo* tegniek is die verteringstyd nie bekend nie en dus kan die *in vitro* rumen- en verteringstyd te kort of te lank wees.

Die resultate het getoon dat die *in vitro* faeces metode van El Shaer *et al*. (1987) ‘n makliker en goedkoper alternatief is vir die klassieke rumenvloeistof *in vitro* metode van Tilley & Terry (1963), soos gemodifiseer deur Engels & Van der Merwe (1967). Die *in vitro* faeces metode gebruik faeces as inokulum en dit is dus ‘n oplossing vir die probleme geassosieerd met die gebruik van gekannuleerde diere. Die gas produksie *in vitro* metode het sekere voordele, maar daar word steeds van gekannuleerde diere vir die verkryging van rumen inokulum gebruik gemaak. Alhoewel die *in vitro* gasproduksie metode van Pienaar (1994) en die sellulase *in vitro* metode gebruik kon word vir die bepaling van OMV% van *Atriplex nummularia* cv. De Kock, was die waardes laer as die *in vivo* bepalings.

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**D** - Digestibility
**DM** - Dry matter
**OM** - Organic matter
**DMD** - Dry matter digestibility
**OMD** - Organic matter digestibility
**DOM** - Digestible organic matter
**DOMD** - Digestible organic matter in the dry matter
**DCP** - Digestible crude protein
**TDN** - Total digestible nutrients
**NDF** - Neutral detergent fibre
**ADF** - Acid detergent fibre
**CP** - Crude protein
**CF** - Crude fibre
**Na** - Sodium
**Ca** - Calcium
**K** - Potassium
**Mg** - Magnesium
**P** - Phosphorus
**N** - Nitrogen
**$CO_2$** - Carbon dioxide
**mg** -milligram
**$NH_3$** -Ammonia
**LW** - Liveweight
**rpm** - Revolutions per minute
**H** - Hour
**µm** - micrometres
**cm** - Centimetres
**mm** - Millimetres
**g** - gram
**kg** - Kilogram

**g/l** - gram per litre

**l** - litre

**ha** - hectare

**ME** - Metabolisable energy

**MJ** - Mega joule

**VFA** - Volatile Fatty Acid

**SCFA** - Short-chain Fatty Acid

# CHAPTER 1

## INTRODUCTORY BACKGROUND ON *ATRIPLEX* SPECIES

The main limitations to animal production in the arid and semi-arid regions, is the scarcity of green forage. In addition, the natural rangeland, which is the basal diet of sheep, is usually overgrazed. Thus, sheep have to survive on a low quality diet throughout most of the year (Ben Salem *et al*., 2002a). There is, therefore, an urgent need for an improvement in feed resources in the arid and semi-arid regions of the country (Aganga *et al*., 2003).

### 1.1 Characteristics of plants

According to Breytenbach *et al*. (1997), *Atriplex nummularia* is a perennial shrub, belonging to the family *Chenopodiaceae*, which is indigenous to Australia and has been found in South Africa for over a hundred years. It can reach a height of three metres (m) and can also develop into bushes of about three metres in diameter. The root system consists of a well-developed taproot with horizontal, lateral roots, and is suberized. The leaves are almost round, blue-grey and covered with a white layer and small saltcells. Male and female flowers are carried on different plants, but are occasionally found on the same plant. The small, round yellow-brown male flowers are borne in clusters, 75 –100 millimetres (mm) in length, on the tips of the branches. Female flowers are grey-green of colour and borne in dense clusters. Pollination is by wind. The seed consists of two leathery, or papery, scale leaves, which have grown together around a small, round red-brown seed. There is a high concentration of sodium chloride (normal salt) in the scale leaves, which inhibits germination. The salt needs to be leached out before germination can take place. There should, therefore, be enough moisture available for the seeds to germinate.

*A. nummularia* is halophytic, or salt loving, and, because of the high salt concentration in the roots and leaves, the cell sap has a high osmotic value. It is this physiological adaptation that makes the plant drought resistant. It also functions very well under high temperatures and high light intensity.

*A. nummularia* is adapted to a wide variety of climatic conditions and also resistant to cold and frost. It will grow on a variety of soil types, even on bare patches of brackish soil and heavy clay soils with poor drainage. The type of soil determines the salt concentration in the plants. Acid and leached sandy soils contain too little sodium- and potassium salts for good production. Plants on brackish soils are, however, less palatable, than those on better soils (Breytenbach *et al*., 1997).

**1.2 Propagation**

To establish *A. nummularia*, it is better to cultivate the plants in a seedbed or holders, and then transplant the seedlings at a later stage, rather than to directly sow the seed in the ground. The seed should be soaked in water for two to three days before sowing, to leach out the salt in the seed coat. The optimum temperature for germination is 15 to 20ºC and the seed must not be planted more than 5mm deep. Sow in August or September and keep the soil moist. When the plants reach the height of 150 to 200 mm, it can be bedded out (Breytenbach *et al*., 1997).

Breytenbach *et al*. (1997) recommends that *A. nummularia* should be established during the late summer or autumn (February or March), under dryland conditions, when the plants are big enough and when there was enough rain. Under irrigation, or when there is sufficient rain earlier in the season, the plants can be bedded out at an earlier age. When the seedlings are bedded out, care should be taken that the roots are not damaged or desiccated. The furrows are made 2 m apart and the plants are planted 1 to 2 m apart in the furrows. Depending on the spacing, 2500 to 5000 plants are required per hectare. Under dry land conditions good establishment can be obtained by the application of 1 litre of water per plant when it is bedded out, followed by two litres ten days later and again two litres 20 days later.

When the plants are well established, seed can be harvested during the second growth season for further expansion of the plantation. The seed can be kept in airtight holders for up to three years. After that germination will decline.

*A. nummularia* can also be propagated by means of cuttings. Young stems should be thicker than 6 mm and 250 mm in length, and planted in sandy soil. Leave a twig or a leaf on the tip of the cutting. Keep the cuttings moist and after six weeks roots will develop and it can be bedded out after a further four weeks (Breytenbach *et al*., 1997).

Breytenbach *et al*. (1997) also recommended that *A. nummularia* should not be grazed in the first season after establishment. After that it could be grazed to encourage the development of a bushy growth form. Plants that are not grazed will grow too tall and sheep can not reach the younger growth. Such plants should be trimmed to a height of 1.5 metres. Sufficient drinking water should be supplied to animals grazing saltbush, because they get thirsty due to the high salt concentrations in these plants. The water should also not contain high levels of salt, because brackish water will inhibit the intake of saltbush.

*A. nummularia* is not a very palatable plant and animals must learn to eat it. It should not be in the same camp as other more palatable plants as this may lead to poor utilisation of saltbush and to overgrazing of the other more palatable plants. Saltbush can be grazed at any time of the year, but rotational grazing should be implemented to give the plants some chance to recover from such defoliation.

### 1.3. Nutritional value of *A. nummularia*

The following tables illustrate the nutrient composition of *A. nummularia*, as reported by several authors.

**Table 1.3.1** The average composition of *A. nummularia* on a dry matter basis (Breytenbach *et al*., 1997)

| Nutrients | % |
|---|---|
| Crude protein | 22.93 |
| Fat | 3.05 |
| Minerals | 19.77 |
| Carbohydrates | 40.58 |
| Fibre | 13.77 |

**Table 1.3.2** Mean crude protein (CP), ash and *in vitro* apparent digestibility (±SEM) of *Atriplex nummularia, A.canescens* and *Cassia sturtii* (Benjamin *et al*., 1995, cited by Aganga *et al*., 2003)

| | | | *In vitro* apparent digestibility | |
|---|---|---|---|---|
| Species | CP(%) | Ash content(%) | DM(%) | OM(%) |
| *Atriplex nummularia* | 18.7(±0.5) | 28.3(±1.4) | 73.5(±1.2) | 58.7(±1.1) |
| *Atriplex canescens* | 17.3(±0.4) | 18.4(±3.3) | 62(±1.3) | 46.7(±1.2) |
| *Cassia sturtii* | 13(±0.3) | 5.2(±0.5) | 50.9(±0.5) | 47.9(±0.4) |

**Table 1.3.3** Mean levels of ash, crude protein (CP), acid detergent fibre (ADF), neutral detergent fibre (NDF), lignin and crude fat (CF) in *Atriplex nummularia* (Watson & O'Leary, 1993, cited by Aganga *et al*., 2003)

| Species | Ash(g/kg) | CP(g/kg) | ADF(g/kg) | NDF(g/kg) | Lignin(g/kg) | CF(g/kg) |
|---|---|---|---|---|---|---|
| *A. nummularia* | | | | | | |
| Cut 1 | 181 | 92 | 337 | 497 | 104 | 22.1 |
| Cut 2 | 247 | 131 | 243 | 405 | 92 | 22.2 |
| Cut 3 | 220 | 91 | 317 | 489 | 93 | 19.8 |
| Cut 4 | 223 | 85 | 306 | 472 | 84 | 22.6 |
| Average regrowth | 230 | 103 | 289 | 455 | 90 | 21.5 |

**Table 1.3.4** Mean levels of sodium (Na), calcium (Ca), potassium (K), magnesium (Mg) and phosphorus (P) and ratios of Na to K (ionic equivalents) in *Atriplex nummularia* (Watson & O'Leary, 1993, cited by Aganga *et al*., 2003)

| Species | Na(g/kg) | Ca(g/kg) | K(g/kg) | Mg(g/kg) | P(g/kg) | Na/K ratio |
|---|---|---|---|---|---|---|
| *A nummularia* | | | | | | |
| Cut 1 | 64.2 | 4.9 | 19.8 | 3.6 | 2.2 | 5.5 |
| Cut 2 | 75.3 | 6.8 | 23.2 | 4.3 | 2.6 | 5.5 |
| Cut 3 | 71.1 | 4.9 | 20.4 | 4.6 | 2 | 5.9 |
| Cut 4 | 68.8 | 4.8 | 17.4 | 4.9 | 1.5 | 6.7 |
| Average regrowth | 71.7 | 5.5 | 20.3 | 4.6 | 2 | 6 |

The stage of growth and maturity has a considerable affect on the nutritive value, palatability and utilisation of *Atriplex spp*. These plants are nutritious in the wet seasons, while they are relatively poor in nutritive value during the dry season (El Shaer *et al*., 2000). The green material usually has a mean value of 74.71% moisture and 25.29% dry matter (Breytenbach *et al*., 1997).

Glenn *et al*. (1998) found that *A*. *nummularia* had a high crude protein and digestible matter content, low fibre and a moderate ash content. Although the crude protein content was somewhat lower than that of alfalfa (10 *vs.* 13%) and the mineral content was higher (15 *vs.* 10%), *A. nummularia* yielded much more crude protein and organic matter, than alfalfa per unit area, due to its high biomass yield. Ben Salem *et al*. (2002a) found that the consumable parts of *A. nummularia* were high in crude protein, fibre and sodium, but relatively low in carbohydrates.

Like most halophytes, the ash content of *Atriplex spp.* is high relative to the non-halophytic species. Because of its high ash content, the energy value of *Atriplex* is low and animals must have a relatively high water intake in order to excrete the ingested salt (Ben Salem *et al*., 2002b; El Aich, 1987, as cited by Aganga *et al*., 2003).

The digestibility of *Atriplex spp.* averaged 59% in spring and 46% in summer. The intake of *Atriplex spp.* varied within the range of 50-55 DM/kg $LW^{0.73}$ (Aganga *et al*., 2003). From Table 1.3.2 it can be seen that *A. nummularia* has a high digestibility and this is due to the salt concentration in the leaves (Benjamin *et al*., 1995, as cited by Aganga *et al*., 2003).

Several methods of feeding *Atriplex spp.* to animals have been investigated. *Atriplex*. *spp.* were either used for a nitrogen (N) supplementation or supplemented with an energy source.

It has been observed, that although sheep maintain their liveweight while grazing *Atriplex spp.* they invariably lose condition (Casson *et al*., 1996, as cited by Hopkins & Nicholson, 1999). This is attributed to a large increase in water intake (Atiq-Ur-Rehman *et al*., 1994, as cited by Hopkins & Nicholson, 1999), to counter the high amount of sodium and potassium salts found in *Atriplex spp.* (Wilson, 1996). Swingle *et al.* (1996), on the other hand, have

shown that *Atriplex* and other halophyte biomass, containing up to 30% mineral content, can replace conventional forages at a 30% inclusion rate in fattening rations for ruminants, with no decrease in weight gain or carcass quality. Valderràbano *et al*. (1996) also found that feeding *Atriplex spp.* did not affect body weight. These researchers suggested that a sole diet of *A. halimus* may be used to maintain sheep and goats, without an energy supplement until animal requirements are higher, due to pregnancy and milk production.

Hopkins & Nicholson (1999) fed saltbush and supplemented it with either hay or grain, to lambs and found that an acceptable meat quality could be produced. The results were compared to lambs fed on lucerne. Based on objective and subjective criteria, it is concluded that fat colour problems are unlikely to occur when lambs are fed on saltbush and that cooked meat taken from lambs grazed on saltbush, have a stronger aroma. But this had no effect on the like or dislike of the aroma by consumers. This conclusion must, however, be restricted to lambs fed for periods up to 68 days and can only be recommended for lambs fed on *A. nummularia*, not other *Atriplex* species (Hopkins & Nicholson, 1999).

The above strategy has real potential for increasing animal productivity on saltbush, as proposed by Warren *et al*. (1990), as cited by Hopkins & Nicholson (1999). Indications are that energy supplements which will increase the metabolisable energy concentration of the diet, will allow greater utilisation of the high levels of crude protein found in saltbush (Atiq-Ur-Rehman *et al*., 1994, as cited by Hopkins & Nicholson, 1999).

Pearce & Jacob (2004) found that meat from sheep grazing saltbush is high in vitamin E. The meat was also leaner and more hydrated, with a consumer appeal equal to that of grain-fed lambs. The higher vitamin E acts as an antioxidant, helping to preserve the fresh red colour and shelf life of the meat.

Hassan & Abd El Aziz (1979) fed fresh leaves and succulent stems of *A. nummularia*, together with 0, 50, 100 or 150 g barley/day to sheep. These authors found that the liveweights decreased during the third week of adaptation, but in the fourth week of the experiment, daily liveweight gains

improved. The CP and CF digestibilities were significantly higher at the highest barley rate of supplementation, and DOM (digestible organic matter) and DCP (digestible crude protein) intakes were significantly improved by barley supplementation.

Abou El Nasr *et al*. (1996) also evaluated the feeding value and utilisation of *A. nummularia* by sheep under arid conditions. *Acacia saligna* and *A. nummularia* were compared in three forms: fresh, air-dried and ensiled. The fresh saltbush diet had higher CP, CF and ash contents than the fresh *Acacia* diet. Processing noticeably changed the contents of CP, ether extract and ash in the saltbush, but not much in the *Acacia*. The DM, CP, ether extract, nitrogen-free extract and neutral detergent fibre fractions were efficiently utilised and digested by sheep given the ensiled shrubs. Saltbush intakes were superior to that of the *Acacia* diet. The most nutritious diet was the saltbush silage, which provided sufficient digested nutrients (TDN and DCP) to meet energy and protein requirements for the maintenance of sheep. The other diets, fresh or air-dried, required supplementation with concentrates to provide a maintenance ration.

Another approach using *Atriplex spp.* was investigated by Ben Salem *et al.* (2002a). These authors used *Atriplex spp*. as a nitrogen-rich supplement, to ensure normal microflora activity in the rumen. It was found that it improved the feeding value of the diets used and consequently sheep growth. The reason why *Atriplex spp*. can be used as a nitrogen-rich supplement lies in the fact that it contains high levels of nitrogen of which a great part is soluble. *A. nummularia* can be regarded as a good, cheap source of nitrogen, which may reduce feeding costs and raise sheep productivity in arid zones (Ben Salem *et al*., 2002a). Improved efficiency of microbial protein synthesis was also found, when Ben Salem *et al*. (2002b) supplied *Atriplex*, either with barley or cactus.

Pregnant Angora goat ewes kept on *A. nummularia* pasture, could maintain pregnancy if they were supplemented with 300 g alkali-ionophore-treated whole maize daily. Such animals, however, ran a relatively high risk of abortion, especially during adverse weather conditions such as cold and wet spells (Hobson *et al*., 1986).

The low tannin content of *A. nummularia* results in rapid and extensive DM and N degradation in the rumen (Ben Salem *et al*., 2002b). Feeding animals with a mixture of shrub species seems to be a recommended practice to dilute the negative effects of possible anti-nutritional factors (tannins, oxalates, etc.) (Ben Salem *et al*., 2002b). The high levels of sodium and nitrogen in *Atriplex*, is most likely to have a diluting effect on the negative effects associated with oxalates (Ben Salem *et al*., 2002b). Norman *et al*. (2004) examined the nutritive value and preference by sheep of two different saltbush species, *A. nummularia* and *A. amnicola* (River saltbush). It was found that the content of total ash and oxalates was present at levels likely to depress voluntary feed intake, especially in *A. nummularia*.

As a supplementary fodder, *Atriplex spp.* should not make up more than 20-30% of the sheep's diet. Casson *et al*. (1996), as cited by Hopkins & Nicholson (1999) suggested that the high salt content of saltland forage plants is likely to be the major determinant of palatability. The dilution of salt, through the availability of other feed resources, would be necessary to improve intake and performance.

*A. nummularia* can carry 20-25 sheep/ha for four months of the year (Breytenbach *et al*., 1997).

### 1.4. Conclusion

*A. nummularia* can be an effective fodder component in mixed diets for livestock. The principal advantages would be that adverse effects due to the high mineral content of the halophyte tissues could be minimised, that animal performance and economic returns might be higher than direct grazing of the shrub species, or in-adequate feed during the dry season in the arid areas. However the main disadvantage of using *A. nummularia* as one of the feed ingredients, would be a reduced feed conversion efficiency, due to the dilution effect of minerals on energy density (Aganga *et al*., 2003).

*A. nummularia per se* is not a balanced diet. The purpose therefore, is not to fatten animals or to get a high performance, but rather to keep the animals from starving during droughts (Breytenbach *et al*., 1997).

Glenn *et al.* (1998) found that *A. nummularia* Lindl. had a higher productivity, water use efficiency and consumptive water use then conventional forage crops in Arizona irrigation districts. The nutritional content of plant tissues was acceptable for use as a ruminant forage. It was concluded that *A. nummularia* had key traits, desired in a plant for the use of saline water, high consumptive use to minimise land area devoted to reuse, high salt tolerance and useful production. It can be concluded, from the above characteristics, that *A. nummularia* can be used in veld reclamation, to cover bare patches and has the potential to be used as a source of feed during droughts.

# CHAPTER 2

## LITERATURE REVIEW OF THE *IN VIVO* DIGESTIBILITY TECHNIQUE

The energy content of most ruminant feeds is very closely related to the digestibility of their nutrients. Although there are other losses of nutrients incurred during metabolism, loss of nutrients in the faeces after ingestion of food is the most important. This is a measurement of digestibility (Kitessa *et al*., 1999). As a nutritive value index, digestibility provides a biological meaningful parameter that can be used in routine feed evaluation. It is also very closely related to metabolisable energy, which accounts for further losses of nutrients via urine and methane. One example of such a relationship allows the metabolisable energy (ME, MJ/kg DM) content of forages to be calculated at 0.0157 times the digestible organic matter (g/kg) in the dry matter ($r^2$ = 0.83, AFRC 1993).

The potential value of a food to supply a particular nutrient can be determined by chemical analysis, but the actual value of the food to the animal can be derived only after making allowances for the inevitable losses that occur during digestion, absorption and metabolism. The first tax imposed on a food is represented by that part of it which is not absorbed and which is excreted in the faeces (McDonald *et al*., 1995).

### 2.1. Calculation of digestibility

The proportion of a feed that is not excreted in the faeces is assumed to have been absorbed by the animal, and this is defined as the apparent digestibility of the feed. It is not the true digestibility, as in ruminant animals the methane that arises from the fermentation of carbohydrates, is lost in eructation and is not absorbed (McDonald *et al.,* 1995). Also, in addition to undigested feed residues, the faeces contain enzymes and other substances secreted in the gut, which are not reabsorbed. Parts of the gut lining, which slough off as the feed passes through the gut, are also excreted in the faeces (McDonald *et al.,* 1995). The amount of this metabolic material that is unavoidably lost, is

directly proportional to dry matter (DM) intake, regardless of forage type (Minson, 1990). Determining the true digestibility rather than apparent digestibility may be scientifically more accurate. However, no advantage has been demonstrated from the use of true digestibility in forage evaluation (Minson, 1990), as the loss of metabolic secretion is a consequence of feeding the forage and should therefore, be accounted for in the assessment of the forage value to the animal.

A variety of means of estimating the digestibility of feeds has been developed (Rymer, 2000).

### 2.1.1 Direct method

To estimate the dry matter digestibility (DMD) of a diet involves the complete collection over a period of time of all the faeces excreted by an animal, once it has been adapted to the diet. The amount of feed dry matter consumed by the animal during this time is also recorded. This is the actual amount of dry matter consumed, and so the difference between the amount of dry matter offered and the amount of dry matter rejected by the animal. The DMD is then calculated from the equation:

**DMD = (DM intake – Faecal DM excreted)/DM intake**

The digestibility of other feed fractions may be determined by substituting them for dry matter in the above equation. For forages, the proportion of digestible organic matter (OM) in the dry matter (DOMD or D value) is often calculated as this provides a measure of the available energy in the forage dry matter. DOMD is calculated using the equation:

**DOMD = (OM intake – Faecal OM excreted)/DM intake**

### 2.1.2 Difference method

If forage is being fed alone, then its digestibility may be determined using the direct method. However, it is often necessary in many situations to supplement the forage with another feed. This complicates the calculation of digestibility somewhat, and there are two main approaches that have been adopted to cope with this situation. In both approaches, it is assumed that there is no interaction between the feeds in terms of their digestibility (Rymer, 2000).

The first approach is to feed the forage with another 'base feed' of known digestibility (McDonald *et al.,* 1995). The digestibility trial is conducted as normal, and the forage (test feed) should constitute the major proportion of the diet.

The DMD of the forage is then calculated using the equation:

**DMD of test feed =**
**(DM intake of test feed – (Faecal DM – Faecal DM from excreted base feed))/**
**DM intake from test feed**

The digestibility of the forage is therefore calculated by difference.

### 2.1.3 Regression method

The second approach is to feed the forage with a supplement (which may be another forage) at a number of different ratios between forage and supplement (Schneider & Flatt, 1975, as cited by Rymer, 2000). The digestibility of each of these different diets is estimated. The digestibility of the diet is then regressed with the proportion of the forage in the diet, and then the digestibility of the forage is estimated by extrapolating to zero inclusion of the supplement (1000 g/kg inclusion of the forage) (Rymer, 2000).

### 2.1.4 Indirect method

In some circumstances, particularly with grazing animals, it may not be possible to measure either feed intake or faecal output, or both, accurately. The digestibility of the forage may still be estimated, if it contains a component that is known to be completely indigestible (Rymer, 2000). This marker should be completely recovered in the faeces and should go unaltered through the gut (Omed, 1986, as cited by Rymer, 2000). If the concentrations of this component in both the feed and the faeces are measured, the digestibility of the forage may be predicted. In this method, complete collections of faeces are not needed, as long as representative samples of faeces are collected. Representative samples of the forage that has been consumed are also required for the analysis of the marker. The equation used to calculate digestibility in this instance assumes that all of the marker consumed is excreted. Therefore, at steady state:

Amount of marker consumed per day = Amount of marker excreted per day.

$$[\text{Marker}]_{\text{feed}} * \text{Feed intake} = [\text{Marker}]_{\text{faeces}} * \text{Faecal output},$$

where marker concentrations are expressed in terms of g/kg DM and feed intakes and faecal outputs are expressed in terms of kg DM/day. Rearranging the above-mentioned equations will result in the following equation:

$$\textbf{Digestibility} = 1 - ([\text{Marker}]_{\text{feed}} / [\text{Marker}]_{\text{faeces}})$$

The marker may be a naturally occurring constituent of the feed, or it may be a chemical mixed into it. However, it is difficult to mix chemicals with forages, although the animals could be dosed with the marker each day, but the disadvantage with this is that it increases the amount of handling that is required and adds to the stress of the animals. Constituents occurring in forages that have been used included lignin, acid-detergent fibre, indigestible acid-detergent fibre (IADF), acid-insoluble ash (AIA) (which is mainly silica) and some naturally occurring *n*-alkanes of long chain length (C-25 – C-35).

The indicator most commonly added to feeds is chromium in the form of chromic oxide, $Cr_2O_3$, although a number of other markers have been used as well. External markers have been found to contaminate grazed forage and

then give biased results in future experiments. This was observed by Sprinkle *et al.* (1995) with chromic oxide.

The indirect, indigestible marker technique is easy to carry out and requires less work than estimating digestibility by the complete collection of faeces. There are three main sources of error associated with this method. These are the estimation of the marker concentration in the forage, secondly the marker concentration in the faeces and thirdly the fact that most markers are not completely indigestible. This third source of error may give rise to very unreliable estimates of digestibility (Rymer, 2000).

Obtaining a representative sample of the forage is also difficult. Grazing animals, particularly sheep, can be highly selective in the forage they consume. One approach that can be adopted is to fistulate the animal's oesophagus. A sample of oesophageal extrusa may then be taken and analysed for the indicator to obtain an estimate of the concentration of the marker in the forage actually consumed (Rymer, 2000).

**2.2 Conducting a digestibility experiment**

An *in vivo* digestibility trial has two stages, the first stage being the adaptation period and the second the collection period. The adaptation period is designed to ensure that a stable population of rumen microflora has been established, that the animals are eating approximately the same amount of feed daily and at the same time the residues being excreted in the faeces arise from the digestion of the feed used in the experiment (Omed, 1986, as cited by Rymer, 2000).

The length of time required for the animal to become adapted to the diet varies from 4 to 12 days (Omed, 1986, Chenost & Demarquilly, 1982, as cited by Rymer, 2000). During this adaptation period, the animals should also be introduced to their digestibility cages if they are being confined (Omed, 1986, as cited by Rymer, 2000).

Following the adaptation period, there is a collection period in which the faeces are collected from each experimental animal. During this collection period, representative samples of the feed are also taken and the amount of feed refused is recorded. Representative samples of the refusals are also taken and analysed, so that the amount of each nutrient that was actually consumed, may be calculated (Rymer, 2000).

The length of time of the collection period varies with the nature of the diet. Shorter collection periods will be required for more uniform diets. A period of 4-12 days is typically chosen (Rymer, 2000).

Complete collection (direct, difference or regression method) or spot sampling (indirect method) of the faeces can be used. With complete collection, the faeces should not be contaminated with urine, and the collection should be made at the same time each day. The faeces taken each day should be bulked over the whole collection period and a sample taken for analysis. When spot sampling is used, samples should preferably be taken twice a day, more frequently if there is diurnal variation in the excretion of the marker (chromic oxide). When the animals are confined, grab samples from the rectum can be taken. This may however, stress the animals. If the animals are grazing, the samples can be taken from the field. The animals can be dosed with coloured dye to identify individual animals, but the dye should not interfere with the digestibility or marker used (Rymer, 2000).

The apparatus used in digestibility trials are metabolism cages, which should have feed boxes that are designed in such a manner that the animals couldn't reach the feed of their neighbour. The walls of the feed box should also be high enough to minimise the amount of feed lost by animals. The inside of the box should be smooth, so that no feed can lodge in crevices. Clean, fresh water should be available, either via an automatic drinking bowl or by a water bucket that is filled two or three times a day. The metabolism cages can have mesh floors as described by Schneider & Flatt, (1975) as cited by Rymer, (2000), which separates the faeces and the urine. The animal stands on a mesh floor, which is situated over a screen that is small enough to retain the

faeces. The faeces collect on this screen, while the urine falls through to a sloped or funnelled pan, which directs it into a collection bottle below. The disadvantage of this type of cage is that the faeces become exposed to contamination by urine and may also be trampled on by the animal. Bags attached to harnesses that are fitted to the animals can also be used. The faeces collects in these bags. Adaptation to the bags for a few days is necessary. The bags work best with wethers and can also be used for grazing animals (Rymer, 2000).

When selecting the animals, the following should be considered:

***Species :*** The advantage of the smaller species is that they consume less feed and are therefore, less expensive to keep. One of the things that should be kept in mind though, is that the digestive tract of such species may be different from the target species (Rymer, 2000).

***Sex* :** Intact male animals are generally preferred, as it is much easier to separate the faeces and urine (Rymer, 2000) .

***Number* :** The number of animals that should be used, depends on the expected difference between test feeds and on the degree of confidence that is required (Rymer, 2000).

If complete collections of faeces are being made, it is important that the amount and composition of the feed that is consumed in the experiment are accurately measured. If the indirect method is used, an estimate of the composition of the forage actually consumed is needed, as it is an accurate estimate of the concentration of the marker in the consumed forage. The level of feeding will depend on what the objective is. With whole forage digestibility the feeding should be restricted to maintenance levels and if selection is practised, the amount offered should be near *ad libitum*. When the forage on offer to the animals in a digestibility trial is prepared, it may in itself not affect the digestibility. The forage offered may be either fed fresh or harvested before the experiment or preserved by drying or freezing it. Alternatively, the animal may be able to graze the forage, and the faeces collected from the field (Rymer, 2000).

## 2.3. Sources of variation of *in vivo* digestibility

### 2.3.1 Particle size

Chaffing, grinding and pelleting can alter particle size of the feed, and consequently its digestibility. Digestibility may be enhanced if the particle size is reduced, presumably by increasing the surface area for enzymatic activity. It can also reduce the retention time. The digestibility may, therefore, also be reduced and thereby reducing the length of exposure to digestive enzymes (Kitessa *et al*., 1999).

### 2.3.2 Level of feeding

Increased intake entails an increase in the rate of passage of ingested food through the gastro-intestinal tract, which shortens the length of time the feed is exposed to enzymatic actions. This may result in depression of digestibility (Kitessa *et al*., 1999). There may also be an interaction between feeding level and associative effects, and between the digestibilities of forage and concentrate when fed together.

### 2.3.3 Associative effects between feeds

An associative effect occurs when, two or more feeds of different digestibility coefficients, is fed in a mixture. The digestibility of the whole mixture is different from the mean digestibility coefficient of the feeds in the mixture. This can be negative or positive (Kitessa *et al*., 1999).

### 2.3.4 Chemical treatment

Formaldehyde treatment of cereal grains, to reduce or prevent rapid fermentation in the rumen, and alkali treatment of cereal by-products, to break the binding of cell wall components to lignin, is the two most common chemical treatments used (Kitessa *et al*., 1999).

### 2.3.5 Miscellaneous

Heat treatment and the species of animal are other factors, which may affect the digestibility of feeds. This cannot be detected by a relationship between the concentration of a chemical fraction in a feed and the digestibility of the feed (Kitessa *et al*., 1999).

## 2.4. Conclusion

Although *in vivo* digestibility is the ultimate measure, it is expensive, labour-intensive, time-consuming and subject to errors. It is also often associated

with the use of digesta flow rate markers, microbial markers and inherent animal variation (Stern *et al*., 1997).

# CHAPTER 3

## LITERATURE REVIEW OF THE *IN SITU* DIGESTIBILITY TECHNIQUE

The Dacron polyester or nylon bag technique has been used widely for estimating ruminal nutrient degradation. It is a relatively simple, low cost method, compared with methods involving intestinally cannulated animals. Another advantage of the technique compared to other laboratory techniques is that it involves the digestive process that occurs in the rumen of a living animal (Stern *et al.,* 1997). It is one of the few techniques that describe the kinetics of feed degradation in the rumen (Adesogan, 2002). The technique can also be readily used in developing countries, since it is not reliant on electricity (Adesogan, 2002).

The procedure is as follows: Samples of dried and milled feed (to pass a 3 mm screen) or wet minced samples are placed in nylon bags (usually 10 x 17 cm). About 2 to 5 g, depending on density, is weighed precisely into each bag. The tied-up bags are incubated in the rumen of sheep on an appropriate diet by suspending them from a rumen cannula. The bags can be inserted into the rumen all together and withdrawn at different time intervals, or the bags can be inserted at different times and withdrawn all at once. This means that all the bags can be washed and dried together. Degradability of dry matter, nitrogen, energy, etc., can thus be measured against time. The pore size of the bags, about 40-60 µm, is such that few particles can escape, and yet micro-organisms can enter the bag. Thus, the fermentation rate inside the bag is similar to that in the rumen. The amount of soluble material in the sample is measured by washing and re-weighing an un-incubated bag. The characteristics of the degradation curve are described by the equation: $P = a + b( 1- e^{-ct} )$, where $P$ is degradation at time $t$, $a$ and $b$ are constants and $c$ is the rate constant of $b$. For protein, the intercept, $a$, is similar to the soluble fraction (washing loss): $b$ represents the potentially degradable fraction (Ørskov, 2000).

McDonald (1981) found that the above equation had to be reassessed when the degradation of roughages was estimated, because of a lag phase. Figure 3.1 illustrates the degradation of a typical roughage.

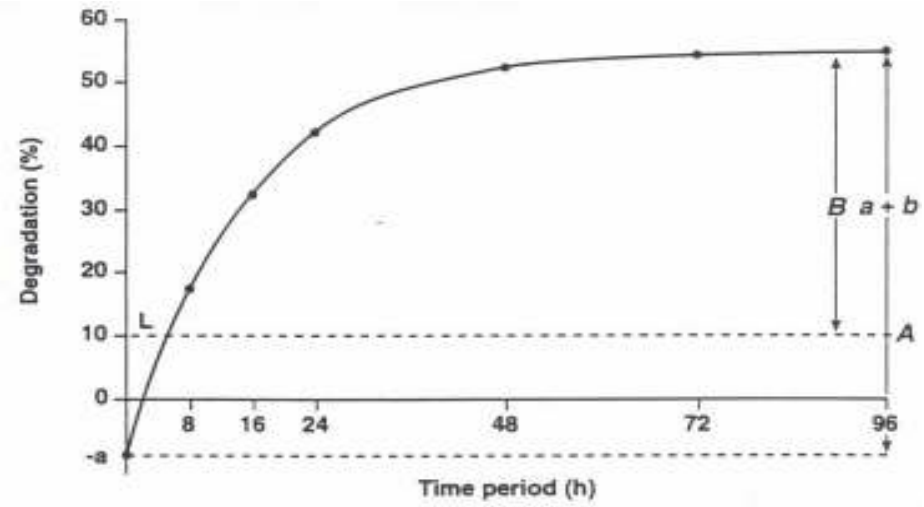


**Figure 3.1** Degradation of a typical roughage diet expressed by the formula $p=a+b\ (1-e^{-ct})$. Due to a lag phase (L), *a* is negative. *A* is the soluble fraction, *B* the insoluble, but potentially fermentable fraction ($B=(a+b)-A$ and *c* the rate constant of degradation (Ørskov, 2000)

When the intercept *a* is no longer similar to the solubility, in fact, when it is most often negative, as there is a lag-phase period, when no nett disappearance of substrate occurs. This is because the microbes take time to adhere to the substrate, so that at first, there is no nett loss in dry matter. In fact, there may be a small increase in dry matter. While it is possible to describe a separate curve for the soluble fraction, there is a problem when increasing the mathematical sophistication. It must be seen in relation to the accuracy required. Ørskov & Ryle (1990), as cited by Ørskov (2000), developed another system, in which the soluble fraction of the sample is determined in the laboratory. The method can use the neutral-detergent fibre (NDF) soluble fraction, the loss after washing with buffer solutions, or the washing loss from a nylon bag containing substrate that was not incubated in the rumen. Since A represents the soluble fraction, the insoluble but fermentable fraction B is worked out as $(a + b)$-A, with *c* as the rate constant.

Particular attention must be given to the choice of incubation times. Ideally, no sample should be taken out of the rumen before the lag phase has ended, normally not before 8 h of incubation. If bags are withdrawn earlier, the curve is distorted and the residual standard deviation (RSD) increases. The other rule is that the asymptote must be clearly identified. This is necessary for accurate description of both B and *c*. This is achieved by ensuring that the differences in degradability between the last two incubation times are small (less than 10% of the second to last incubation), i.e. 40 and 44%. Usually 72 or 96 h are sufficient to describe the asymptote clearly.

All feed evaluation systems do not predict intake, and this is a particular problem when roughages or forages are grazed or stall-fed *ad libitum*. Ideally, feed evaluations should provide farmers with an exchange rate of one feed compared with another. They should allow planners of livestock production to assess the possible production and predict export, or necessity for import of feed, etc. Digestibility and metabolisability, while positively correlated with feed intake, can generally only account for about half of the variation in feed intake (Ørskov, 2000).

If feeds can be characterised in such a way that potential feed intake is predicted, progress in roughage evaluation will be made. Intake is influenced by interactions between feed and animal, especially those that affect ruminal retention of fibre, such as, particle reduction, outflow of small particles and rumen volume. A feed evaluation system must characterise the feeds themselves, as the animal interaction cannot be determined in each instance. Also, because of the length of time it takes, it would be very expensive and impractical to determine intake and utilisation of each feedstuff by feeding trials (Ørskov, 2000).

In many experiments, the characteristics of feed degradability have been described by the following fractions. The soluble fraction *A*, which, arises from cell contents, takes up little space and is 100% fermentable. The insoluble, but degradable fraction *B*, which takes up space, as long as it remains in the

rumen undegraded. And *c*, which is the speed at which the *B* fraction is degraded. The undegradable fraction, 100 – (*A*+*B*), takes up space until it is eliminated by outflow from the rumen. All these fractions have an influence on the volume of ingesta in the rumen. These rate constants have been used in an attempt to develop a system that can predict not only feed nutritive value, but consumption as well. Multiple regressions were tested to see if intake and digestibility could be predicted from values for *A*, *B* and *c*. It was found that *A*, *B* and *c* separately gave the best estimates of intake, because the correlation's between them were weak (Ørskov, 2000).

The *in situ* technique can also be used for studies to evaluate the rumen environment. When the technique is used to evaluate feed, the rumen environment should be optimal so that maximum degradability of the feed is expressed. However, factors like acid conditions and negative interactions between feeds or deficiencies in the diets, e.g. of N (nitrogen) or S (sulphur), can make the rumen environment sub-optimal. As these influences are usually reflected in cellulolysis, the study of the rumen environment is centralised in changes in the rate constant, *c*. By using a standard feed substrate and varying the rumen environment, the feeding regime can be studied and the most cost-effective rumen environment can be identified, as shown in Figure 3.2.

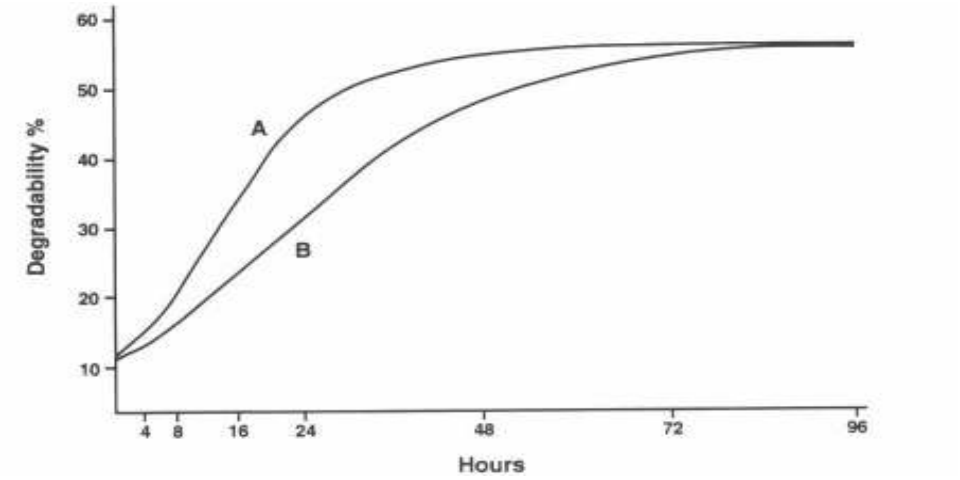


**Figure 3.2** Degradation of a roughage diet in a rumen in which the rumen microbial environment is optimal (A) and suboptimal (B). Note intercept an asymptote are similar (Ørskov, 2000)

The *in situ* method, can also be used, to identify optimal addition of N to a low N diet and to identify the optimal content of easily fermentable fibre in the diet (Silva & Ørskov, 1988, as cited by Ørskov, 2000). It is also possible to identify the season, in which to supplement pasture-based diets to be most cost-effective (Ørskov, 2000).

The *in situ* or *in sacco* method, though not an *in vitro* method, plays a vital role in measuring the rate of degradability of individual nutrients in sections of the gastro-intestinal tract. It is less commonly used for measuring total tract DMD. When used to predict total tract *in vivo* DMD, it has, however, usually been more accurate than other *in vitro* methods (Kitessa *et al*., 1999). The most common use is to estimate microbial protein degradation in the rumen (Stern *et al*., 1997).

Statistical or experimental design and replication should be considered when using the *in situ* method. Usually, all the bags were placed in the rumen together and then withdrawn at different times. Sometimes it is more practical to put the bags in at different times, and withdraw them all at once. Incubation

times can also be flexible. The particle size of the sample should be such to avoid small particles being lost from the bags (Ørskov, 2000).

The replication adopted will depend on the objectives of the study. If the feed is evaluated and the results are to be used in diet formulation or the rumen environmental conditions are to be estimated, the variation between animals should be considered and more than one cannulated animal should be used. If the objective is to rank the feed potential of forages, then only one animal may be used (Ørskov, 2000).

The *in situ* method entails many sources of variation that may confound the comparison of results, both within and between laboratories (Kitessa *et al*., 1999). These sources of variation include the bag characteristics, sample preparation, weighing of the samples, and placement in rumen, post-rumen incubation processing, host diet effect and animal effects.

**Table 3.1** Factors affecting the accuracy of the *in situ* rumen degradability (Adesogan, 2002)

| **Factor** | **Effect** | **Reference** (as cited by Adesogan *et al*., 2002) |
|---|---|---|
| Oven drying | Reduces N degradability and solubility | (Lopez *et al*., 1995) |
| Freeze drying | Enhances particulate losses, but is better than oven drying methods for silages | (Vik-Mo, 1989; Lopez *et al*., 1995) |
| Grinding/pre-wetting samples | Underestimates the lag phase and overestimates degradation rates due to increased microbial colonisation | (Noziere & Michalet-Doreau, 2000) |
| Particle size | Larger particles prolong the lag phase | (Emmanuele & Staples, 1988) |

| | | |
|---|---|---|
| Washing procedure | Overestimates soluble and particulate losses, but is less subjective than hand washing | (Cockburn *et al*., 1993) |
| Particulate losses | Overestimates rumen soluble content and the extent of degradation, but underestimates degradation rates if the particles lost would have degraded rapidly | (Emmanuele & Staples, 1988) |
| Incubation sequence | Reverse sequence incubation can reduce degradation rates due to interruptions and differences in rumen environment of samples incubated for different periods | (Nocek, 1985) |
| Incubation site | Substrate incubation in the dorsal sac underestimates degradability due to lower colonisation rates than those in the ventral sac | (Stewart, 1979) |
| Bag pore size | If < 15µm can reduce degradation, by restricting microbial colonisation and diversity and by trapping fermentation gasses. If > 40µm, causes losses of insoluble/undegradable particles | (Huntington & Givens, 1995) |
| Bag weave size | Monofilamentous cloth's pores are prone to stress-induced distortion, which can enhance particulate | (Marinucci *et al*., 1992) |

| | | |
|---|---|---|
| | losses. Multifilamentous cloth is different. | |
| Microbial contamination of residues | Underestimates N degradation in low N feeds. Removal methods can be expensive, laborious or inaccurate | (Olubobokun *et al*., 1990) |

**Conclusion**

Broderick *et al.* (1991), as cited by Stern *et al*. (1997) emphasised that, although the *in situ* technique is imperfect in ways that cannot be fully compensated for, it is rapid, fairly reproducible, and requires minimal apparatus. The technique has advanced the knowledge of protein metabolism in ruminants and is a valuable tool for assessing the kinetic parameters of feed degradation (Adesogan, 2002). However, this technique requires surgical preparation of an animal with a ruminal cannula and facilities for animal maintenance, which may be inconvenient and expensive.

# CHAPTER 4

## DIFFERENT LABORATORY TECHNIQUES TO DETERMINE THE DIGESTIBILITY OF *ATRIPLEX NUMMULARIA* CV. DE KOCK.

### GENERAL INTRODUCTION

The development and application of laboratory methods for predicting the nutritive quality of forages have been an active and successful area of research during the latter half of this century (Jones & Theodorou, 2000). Many attempts have been made to predict the intake and digestibility using laboratory techniques, and thereby developing regression equations to predict digestibility from forage chemical composition (Getachew *et al.,* 1998).

Traditional wet chemistry techniques for estimating chemical components to predict digestibility of feeds, rely on a statistical association between the concentration of a chemical fraction in a feed and the digestibility of the feed *in vivo* (Kitessa *et al*., 1999). This association can be a simple correlation or a direct cause-effect relationship (Weiss, 1993, as cited by Kitessa *et al*. 1999).

It is generally accepted that feed fractions with the greatest influence on digestibility, are those that constitute the cell wall fraction. Cell contents are almost entirely digestible. The most common cell wall constituents used in predictive equations for digestibility are neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin. The accuracy in predicting *in vivo* digestibility from fibre fractions varies, both within and between the fractions used (Kitessa *et al*., 1999).

Theoretically, the error of prediction should decrease, as the fibre fraction is progressively refined to its most indigestible constituent, from NDF to ADF to lignin. However, this is not always the case, and some feeds are very low in lignin, especially grain supplements. Ranking such feeds for digestibility, or energy content, is not of much value (Kitessa *et al*., 1999).

The accuracy of prediction equations is improved when the equations are applied within the feed types used to generate the equations, because the equations have an inherent population-dependence (Weiss, 1993, as cited by Kitessa *et al*., 1999).

The greatest sources of variation in accuracy of prediction, are the extrapolations across feed types and difference in the chemical fraction chosen. In addition, differences in the methods used to determine the concentration of chemical fractions, can lead to between laboratory variations in the prediction of *in vivo* dry matter digestibility (DMD) from chemical fractions. Such variations can be overcome by standardising methods of chemical analysis (Kitessa *et al*., 1999).

Kitessa *et al*. (1999) stated that, the advantage of using equations based on chemical indices is that the determination of chemical components is simple, rapid and cheap, but the statistical association between chemical indices and digestibility, doesn't represent the biological processes involved in food digestion.

An important development has been the introduction of biological methods (Jones & Theodorou, 2000). Three major digestion techniques (biological methods) that simulate the digestion process are currently available to determine the nutritive value of ruminant feeds:

1. Digestion with rumen micro-organisms as in the work of Tilley & Terry (1963) or the gas method of Menke *et al.,* (1979). Digestion with faeces micro-organisms (El Shaer *et al*., 1987), is an alternative to rumen fluid inoculum.
2. Cellulase methods
3. *In situ* incubations of samples in nylon bags in the rumen.

Biological methods are more meaningful, since micro-organisms and enzymes are more sensitive to factors influencing the rate and extent of digestion, than are chemical methods. However, some important points need

to be addressed in the development of a viable *in vitro* technique. An efficient laboratory method should be reproducible and should correlate well with actually measured *in vivo* parameters. *In vitro* methods have the advantage of not only being less expensive and less time-consuming, but they also allow one to maintain experimental conditions more precisely than can be done in *in vivo* trials (Getachew *et al.,* 1998).

Because of increased public scrutiny, elicited by the animal rights movement, the use of invasive surgical procedures for nutritional research has become more difficult to justify. *In vivo* measurement of nutrient digestion is expensive, labour-intensive, time-consuming, and subject to error associated with use of digesta flow rate markers, microbial markers, and inherent animal variation. Hence, alternative methods for predicting nutrient digestion of dietary feed ingredients in the rumen and small intestine are needed (Stern *et al.,* 1997).

The objective of this study was to determine the digestibility of *A. nummularia* cv. De Kock (Oldman's saltbush), supplemented with two energy sources, maize and barley. This was done by using different *in vitro* techniques, and comparing the results with the *in vivo* organic matter digestibility data, to determine, which *in vitro* digestibility technique, provides the best prediction of the organic matter digestibility (OMD).

The following review of literature will be of the individual *in vitro* techniques.

## 4.1 THE EVALUATION OF THE *IN VITRO* RUMEN LIQUOR DIGESTIBILITY TECHNIQUE

An excellent example of a systematic approach to the establishment of a feed evaluation system, was the development of the first routine *in vitro* system by Tilley & Terry (1963). Recognising the importance of diet digestibility, as an index of nutritional value, these workers established a comprehensive *in vivo* database of forage digestibilities, from which, suitable predictive procedures could be developed. A wide range of forages, both grasses and legumes, were systematically fed to mature sheep at maintenance levels to obtain measurements of whole-tract digestibility. Subsequently, these feeds were

subjected to an *in vitro* technique, comprising two stages, to simulate ruminal and postruminal digestion. A series of equations to predict whole-tract digestibility for different diets, were proposed (Beever & Mould, 2000).

The establishment of the technique led to the development of the concept of forage digestibility (D), or DOMD, value, defined as the content of digestible organic matter in forage DM, and equations to predict DOMD (%). Estimates of D values, have also been used in systems of grassland management, to optimise the use of grazed grass or the stage of harvesting of grass for ensiling. Plant breeders also adopted this concept, as a screening procedure to examine the nutritional value of new forage cultivars. Equally, DOMD values derived from IVD (*in vitro* digestibility) estimates have been used to predict forage ME contents. A value of 0.15 has been adopted to convert DOMD to ME (i.e. ME (MJ/kg DM) = 0.15DOMD%) for fresh and dried grass hay. A higher coefficient of 0.16 was used for high-protein legumes and fermented feeds (Beever *et al*., 1999, as cited by Beever & Mould, 2000). Estimates of forage IVD have also been used to predict forage intake – yet another example of the utility of this index of nutritive value (Beever & Mould, 2000).

The technique first described by Tilley & Terry (1963) has been the most commonly used *in vitro* method for predicting digestibility and as a selection tool for improving the nutritional quality of forages (Stern *et al*., 1997).

This two-stage *in vitro* technique of Tilley & Terry (1963) has shown that it gives results well related to *in vivo* digestibility, for a wide range of forages and under a variety of growing conditions. However, there are inherent problems in the use of micro-organisms in rumen fluid for forages (Jones & Theodorou, 2000). Maximising *in vitro* digestion depends on several factors, including dilution of the ruminal inoculum, type of buffer used, particle size of the sample, type of mill used for grinding, and type of diet the donor animal is fed (Stern *et al*., 1997).

### *4.1.1 Activity of inoculum*

The activity can vary, depending on time of collection after feeding, diet of the donor animal, species of donor animal, and animal-to-animal variation within a species (Kitessa *et al*., 1999).

Jones & Theodorou (2000) found that this activity differs, between different donor animals and in the same animal on different days. Freeze-drying, or other methods of preservation, altered the microbe activity. Fresh rumen fluid should, therefore, be available and this entails, having cannulated sheep or cattle available as donor animals. The activity of the microbial inoculum should be checked each time by incubating two or more standard samples of known digestibility.

An optimum time to collect rumen fluid would be 8-12h after feeding (Weiss, 1994, as cited by Kitessa *et al*., 1999). This is because the activity of most fibriolytic enzymes peaks at this time according to Williams (1988), as cited by Kitessa *et al*., 1999. If practical, feeding at least 3 times per day can reduce the variability (Weiss, 1994, as cited by Kitessa *et al*., 1999). Another influence on the potency of the rumen fluid is the consumption of water (Jones & Theodorou, 2000).

### *4.1.2 Processing of rumen fluid*

Ruminal inoculum is typically strained through several layers of cheesecloth and diluted (20:80) in saline solution, artificial saliva, or various buffers and this will affect the microbial concentrations (Stern *et al*., 1997). Craig *et al*. (1984), as cited by Stern *et al*. (1997), suggested, that when simulating ruminal fermentation of fibre from different feedstuffs, strained ruminal fluid, plus an inoculum of particulate-associated bacteria should be used, since it is more effective than strained ruminal fluid alone.

### *4.1.3 Diet of donor animals*

The donor animals need to be maintained on a standard feeding regime for long periods to minimise variability in inoculum potency. The feeding and care of the animals is a high and costly labour input (Jones & Theodorou, 2000).

Forages under test are not usually the same as the maintenance diet of the donor animals, thus affecting the rumen microorganism population (Zacharias, 1986).

When the rumen-liquor technique is used to predict the digestibility and energy value of feeds, such as straw, which is generally bulky and of very low quality, it is possible that the 48h incubation may be insufficient. The microbial population may gradually shift to highly fibrolytic species when animals are retained on straw diets (Kitessa *at al*., 1999). Cone *et al.* (1989), as cited by Stern *et al*. (1997), demonstrated that the type of diet fed to the animal had an effect on the results of the *in vitro* technique. *In vitro* starch disappearance was greater, when ruminal fluid was obtained from a concentrate-fed cow than when it was obtained from a hay-fed cow. A good practice for a laboratory would be to maintain donor animals on a low cost feed that is similar in quality characteristics to feed samples commonly analysed in that laboratory (Kitessa *et al*., 1999).

Engels & Van der Merwe (1967) also studied the effect of the ration fed to the donor sheep. These researchers found that the differences between *in vivo* and *in vitro* digestibilities of OM were greatest when rumen liquor was obtained from sheep on an oat hay diet, than when the sheep were fed on a lucerne diet. Highly significant differences were observed (p=0.01) for rumen liquor from different sources. Differences appear to be more pronounced with forages of low crude protein content, such as oat & grass hay. It was found that there was no significant variation in *in vitro* digestibility values, obtained for the same forages between different sheep. The individuality of donor sheep fed on the same ration didn't have a significant effect.

### *4.1.4 Collecting and handling of rumen fluid*

Maintaining anaerobic conditions, the high viscosity of digesta that influence filtration, unpleasant smells and a need for hygiene, to avoid possible risk of infection from pathogens, add to the analytical difficulties of using rumen fluid *in vitro* studies (Jones & Theodorou, 2000). Zacharias (1986) also found it difficult to collect and handle the rumen fluid. Grant & Mertens (1992b) tested several factors that could affect fibre digestibility values, using the *in vitro*

technique, including the maintenance of anaerobic conditions. Purging tubes with $CO_2$, but not gassing continuously, resulted in a 56% increase in lag time for NDF digestion and a 69% decrease rate of NDF digestion. These authors also suggested the use of nutritional additives, such as micro-minerals, vitamins and tryptone, to ensure that non-fibre factors did not limit fermentation, especially with substrates low in protein and micro-minerals.

An *in vitro* buffering system, capable of pH control between 5.8 and 6.8, has been developed by Grant & Mertens (1992a) and successfully used to study fibre digestion *in vitro* (Grant & Mertens, 1992c). Starch digestion also seems to be affected by the pH *in vitro* (Stern *et al*., 1997).

Richards *et al*. (1995) recommended that dilutions of 1:2 or 1:3 be used when studying starch digestion of grains that are rapidly fermented. A 1:1 dilution showed a lower rate and extent of starch digestion, because of a lower pH when the ruminal fluid was less diluted.

### *4.1.5 Sample preparation*

Richards *et al.* (1995) found that the type of mill used to grind the sample also had an affect on *in vitro* digestibility. Greater starch digestibilities were noted when a Udy mill, compared to a Wiley mill, was used for milling samples. Screen size might also affect digestibility, but samples ground to pass through 2 mm and 1 mm screens yielded similar starch digestibilities. Cone *et al.* (1989) reported increasing rates of starch disappearance when particle size decreased from 1 to 0.1 mm. Kitessa *et al*. (1999) suggested that, variations between and within laboratories are minimised if samples are ground to a standard mesh size.

### *4.1.6 Type of animal*

The effect of species of donor animal on the accuracy of *in vitro* DMD has been equivocal. The differences are small and keeping and caring for the animals, are likely to determine the species (Kitessa *et al*., 1999). By keeping a few cannulated animals, the rumen fluid collected can be bulked to overcome the animal-to-animal variation (Gulati *et al*., 1997, as cited by Kitessa *et al*., 1999). Zacharias (1986) also suggested that, if there is a

difference between the type of donor animal (usually sheep) and those to which the research applies, it could be a potential area for error.

Most of the problems due to the variability in the quality of the rumen fluid can be prevented by including standards of known *in vivo* digestibility in experiments (Jones & Theodorou, 2000; Adesogan, 2002). This allows for correction of the results relative to standard values, but in practice, it is often difficult to make a valid correction, unless a large number of standard samples are included in each analytical batch (Jones & Theodorou, 2000).

Although the above problems are not insurmountable they provide a number of areas for potential error, especially for the unskilled analyst or range scientist, who conducts his own forage analysis (Zacharias, 1986).

The more complicated shortcomings of the technique include the disregard of post-gastric digestion, the outflow of digesta and the digestion of pepsin-insoluble nitrogenous compounds. Poor predictions of *in vivo* digestibility, because of these factors have been reported (Adesogan, 2002). Ibbotson (1982), as cited by Adesogan (2002), observed a poor prediction of N digestibility, because of Maillard products in silages. These products are digested *in vitro*, but not *in vivo* and this resulted in an underestimation of metabolic faecal N *in vitro*.

### 4.1.7 Modifications

Several modifications of the original procedure have been used to maximise the digestion process, because *in vitro* systems that do not maximise digestion kinetics, may not detect differences in substrate digestion (Grant & Mertens, 1992b).

The original Tilley & Terry (1963) method involved two stages in which forages were subjected to 48h fermentation in a buffer solution containing rumen fluid, followed by 48h of digestion with pepsin in an acid solution (Getachew *et al.,* 1998). With the passage of time, possible alternatives to this *in vitro* system, as proposed by Tilley & Terry (1963), have been suggested.

Several workers have found that the rumen fluid-pepsin technique gives less accurate predictions of digestibility in silages and straw, than in fresh grasses (Givens *et al.*, 1995; Adesogan *et al.*, 1998). Hence, to better account for post-ruminal digestion, Van Soest *et al*. (1966), as cited by Adesogan (2002), modified the method, introducing a final washing procedure after the 48h incubation with neutral detergent solution replacing the acid-pepsin step in Tilley & Terry (1963) to estimate the predicted true digestibility.

Gizzi *et al.* (1998) evaluated a rapid *in vitro* technique, to study the activity of rumen micro-organisms and to estimate the nutritive value of feeds. The rumen culture apparatus used is essentially a batch system, modified in order to ensure conditions of strict anaerobiosis and the balanced growth, of all the microbial forms present in the mixed rumen inoculum. *In vivo* and *in vitro* studies were done simultaneously and by comparing them, validation of this technique was obtained. Microbial flora composition, biochemical parameters of rumen fluid and feed degradability were analysed. It was found that the total viable bacteria count, did not show significant differences between fermentations, *in vivo* and *in vitro*, at any of the incubation times. The dry matter degradability values found *in vitro* were similar to those *in vivo*, except for the immediately soluble fraction. This fraction represents the feed degraded at 0h, which is the amount of dry matter solubilised, after washing the nylon bags in water without submitting it to any fermentation process. It was significantly lower ($P<0.05$) *in vitro*. Less variability was associated with results obtained from *in vitro* system compared with measurements in animals. Ammonia nitrogen and degradability of compound feed nitrogen did not show significant differences between the two systems.

Engels & Van der Merwe (1967) tested the Tilley & Terry (1963) *in vitro* technique for the evaluation of South African forages using rumen liquor from different sources. These reasearchers found highly significant differences ($P = 0.01$) in the *in vitro* digestibility of the same samples, when it was obtained using rumen liquor derived, either from a sheep on lucerne hay or from sheep on oat hay. The *in vitro* method yielded results with a high degree of repeatability. No significant differences ($P=0.5$) were obtained with *in vitro*

digestibility of organic matter, when rumen fluid from four different sheep, on the same ration, was used as inoculum.

A slight modification, regarding N supplementation, was also investigated. Urea was added at different levels ranging from 1 –60 mg. It was established that with urea, which contains 46% N, to obtain 1mg of N an addition of 2.17 mg of urea was necessary. When 20 mg N was added to the rumen liquor, obtained from a sheep fed on oat or lucerne hay, no significant difference (P = 0.05) was observed. The highest *in vitro* digestibility was obtained with the addition of 20 mg N. With more than that, digestibility declined. This is thought to be due to the toxic effect of excess ammonia ($NH_3$).

Engels & Van der Merwe (1967) found that the reason for the low protein forages with the higher *in vivo* digestibility could be due to the higher N in the rumen liquor from the recycling of urea via saliva and the rumen wall. Sheep on lucerne hay showed no effect on legumes with N supplementation, but on non-legumes there was an effect. With the rumen fluid of sheep on an oat hay diet, N supplementation improved *in vitro* digestibility in all samples. The difference in N content of the two different rumen liquors may be the reason for the differences in digestibilities.

### 4.1.8 Advantages & Disadvantages

The main advantage of this method is that it is less susceptible to factors, which confound the prediction of digestibility from chemical indices. It has the capacity to account for factors such as heat treatment, associative effects, alkali treatment of straws, and species of animal (if rumen liquor is gathered from the same species as the test animals). For instance, associative affects on the digestibility of a forage fed with concentrates can be overcome by feeding donor animals the forage: concentrate ratio planned, to the test animals (Kitessa *et al*., 1999).

Engels & Van der Merwe (1967) investigation showed that the Tilley & Terry (1963) *in vitro* digestibility technique yielded a high degree of repeatability. The *in vitro* OM digestibility was determined with rumen liquor from the same sheep drawn on three different days. It was found that the *in vitro* results were highly repeatable from day to day and even in the next year.

The Tilley & Terry (1963) *in vitro* method appears to have several disadvantages. The method is an end-point measurement (gives only one observation) thus, unless lengthy and labour-intensive, time-course studies are made, the technique does not provide information on the kinetics of forage digestion and the residue determination destroys the sample and a large number of replicates are, therefore, needed. The method is, therefore, difficult to apply to materials such as tissue culture samples, or cell wall fractions (Getachew *et al*., 1998). The requirement of donor animals to supply rumen fluid, which entails the cost of acquiring the animals, surgical costs, labour for animal care, feed cost and welfare issues, is also a disadvantage. The technique is not able to accommodate the effect of change in particle size, because the samples are ground through the same screen size. The technique is also very slow, in that at least two days are required per batch of samples (Kitessa *et al*., 1999).

## 4.2 THE EVALUATION OF THE *IN VITRO* FAECAL LIQUOR TECHNIQUE

Rumen fluid inoculum is required in most of the *in vitro* techniques utilising a microbial fermentation approach to feedstuff evaluation (Tilley & Terry, 1963; Menke *et al*., 1979). The necessity for cannulated animals to provide this inoculum raises a number of practical problems, e.g. surgical facilities, constant care to avoid infections and costs associated with the long-term maintenance of these animals. In addition, there are a number of ethical considerations regarding these techniques with respect to animal welfare (Mauricio *et al*., 2001).

The prime function of the rumen liquor is to provide a rich source of fibrolytic organisms, which leaves open the possibility that these might be more readily obtained elsewhere (Omed *et al*., 2000). Gut micro-organisms, which are closely associated with plant debris in the rumen, are also excreted with plant residues in the faeces (Van Soest, 1982; Theodorou *et al*., 1993, as cited by Omed *et al*., 2000). The faecal material also remains largely anaerobic after

voiding and the microflora can be viable for several hours after excretion from the digestive tract (Holter, 1991, as cited by Lowman *et al*., 1999).

Faeces can, therefore, be a cheap, readily available source of micro-organisms, which do not require the use of surgically prepared (cannulated) animals. It can be collected from any individual or several animals, thereby, minimising the effects of animal to animal variation (Lowman *et al*., 1999). Thus, faeces may be an alternative to the rumen fluid inoculum techniques.

Balfe, (1985), as cited by Omed *et al*. (2000) first explored the use of faecal organisms, for the estimation of the digestibility of forages. A filtered homogenate of sheep faeces in artificial saliva, instead of rumen liquor, was used in the modification of the Tilley &Terry (1963) method. The method was not convenient for batch operation, as the faecal liquor rapidly blocked the sintered filters, a feature confirmed by El Shaer *et al*. (1987) and Akhter *et al*. (1994). The undigested residues were recovered by centrifugation, after transfer of the liquor from the fermentation tubes. Nsahlai & Umunna (1996) also used reconstituted sheep faeces as inoculum to determine *in vitro* digestibility and reported a coefficient of correlation of 0.88 between DM digestibility obtained using reconstituted faeces, and DM digestibility obtained using ruminal fluid.

### 4.2.1 Factors affecting the activity of faecal liquor

#### *4.2.1.1 Source of faeces*

Several researchers used different animal species faeces as a source of inoculum. When comparisons have been made between liquors from different species, sheep faeces have been consistently shown to provide the greatest fibrolytic activity and should, therefore, be considered the preferred faecal source (Omed *et al*., 2000). Aiple *et al*. (1992) concluded that the pellet form of sheep faeces provides a more stable environment for the micro-organisms to survive and those results, with faeces from sheep, are definitely superior compared to those obtained using faeces from cattle and pigs. Even faeces from donkeys have been used as a source of faecal inoculum. Kirkhope &

Lowman (1996) have suggested that the use of such an inoculum could prove useful in assessing nutritive value for donkeys and ponies.

Lowman *et al*. (1999) and El-Meadaway *et al*. (1998) used faeces as microbial inoculum in gas production studies. Lowman *et al*. (1999) concluded that equine faeces could also be a suitable source of microbial inoculum. Equids are hindgut fermenters, with little or no post fermentative digestion and absorption of microbial cells, as in the case of ruminants. El-Meadaway *et al*. (1998) suggested that faeces from cattle are an effective inoculum, particularly for good quality feeds.

#### *4.2.1.2 Effect of diet*

El-Shaer *et al*. (1987) noted that faeces obtained from sheep fed hay or equal weights of hay and concentrate produced similar results. This agrees with the findings of Akhter *et al*. (1994) who used bovine faecal liquor, prepared from concentrate-, concentrate-and-hay or hay-fed donors. No significant differences were found. The dietary effect on faecal liquor activity was less than that observed when using rumen liquor (El Shaer *et al*., 1987).

Food residues reaching the large intestine are thoroughly leached, leaving mainly residual roughage. Data suggest that a high fibre dietary component is necessary for donor animals, thus ensuring that sufficient fibrous residue is present in the hindgut to sustain an active cellulolytic bacterial population. Concentrates, within the diet, would not be expected to influence *in vitro* digestibility results (Omed *et al*., 2000).

Aiple *et al*. (1992) on the other hand, studied gas production, using a variety of feeds with liquor from faeces obtained from animals fed different basal diets. Small but significant differences were identified. For a dense and active microbial population in the hindgut and hence in the faeces, the diet of the animals should provide enough fermentable substrate for the lower digestive tract (Aiple *et al*., 1992). It seems important to formulate the diet for the donor animals as manifold as possible (Aiple *et al*., 1992). Mauricio *et al*. (2001) also suggested that when comparing rumen liquor and faeces, data from similar types of forage should be used when establishing relationships, because the type of feed offered will have a major influence on the respective microbial populations.

#### *4.2.1.3 Age of faeces*

Initial studies on faecal liquor (El-Shaer *et al.,* 1987) used ovine faecal pellets within 1h of voiding, on the assumption that the pellets would briefly maintain a suitable environment for cellulolytic bacteria. However, it was noted that faeces voided up to 6h previously, were equally effective for forage digestibility (El-Shaer *et al.,* 1987). More recently Pilling (1997), as cited by Omed *et al*. (2000), established that ovine faeces maintained their cellulolytic capabilities for up to 24h, when stored either at room temperature or at 5°C under anaerobic conditions. Storage for 24h at room temperature did however, result in a significant decline in activity. Aiple *et al*. (1992) found that gas production from hay but not from concentrates by faecal liquor was reduced, following storage of faeces for 1h under aerobic conditions at room temperature, indicating the greater sensitivity of cellulytic organisms than non-cellulolytic organisms to storage. Akhter *et al*. (1995) concluded that fresh, frozen or freeze-dried cow faeces are satisfactory and repeatable substitutes for rumen liquor as sources of micro-organisms for *in vitro* digestibility assays of forages. Nsahlai & Umunna (1996) found that the faeces voided up to 2h prior to inoculum preparation, produced a significant correlation of *in vitro* faecal liquor digestibility ($r^2$ =0.85) with *in vitro* rumen liquor, but that this was lower than that of other investigations using fresh faeces.

It can be concluded that the method does not depend on fresh faeces, although these are the most effective (Omed *et al*., 2000).

#### *4.2.1.4 Choice of buffer*

El-Shaer *et al*. (1987) successfully used artificial saliva (McDougall, 1948), which is a bicarbonate buffer. The disadvantage of using this buffer is that when it is dissolved the natural pH is 8.23, and gassing with $CO_2$ is needed to achieve the pH of 6.8 favoured by cellulolytic bacteria. This is a time-consuming procedure and direct acidification, following completion of the fermentation stage, causes extensive frothing. It is therefore necessary to physically separate the residue from the inoculum, prior to second-stage acid digestion (Omed *et al*., 2000).

Menke's medium (Menke *et al*., 1979), or a modified version has been used when investigating gas production (Aiple *et al*., 1992). A phosphate buffer (Pigden, 1969) was used by Solangi (1997), as cited by Omed (2000). Gas production from a bicarbonate buffer is higher than with a phosphate buffer, since a bicarbonate buffer reacts with the acid products of fermentation to release $CO_2$.

#### *4.2.1.5 Separation of bacteria*

The bonds that are formed by the cellulolytic organisms with their substrates have to be broken, before effective faecal inocula can be produced from ovine faeces (H.M. Omed, unpublished observation, as cited by Omed *et al*., 2000). Solangi (1997), as cited by Omed *et al*. (2000), found that the percentage of cellulose samples digested during 48h incubation was greater when the faeces and artificial saliva were pummelled together in a plastic bag (75%) or ground together in a pestle and mortar (70%). When shaken with glass beads in a bottle (67%) or homogenised in a food processor (65%), the percentage digested declined.

#### *4.2.1.6 Faeces-to-buffer ratio*

El-Shaer *et al.* (1987) used 60 g of faeces in 300 ml of artificial saliva (McDougall, 1948). El-Meadaway *et al.* (1998) used a faecal suspension, with 3%, 6% and 9% fresh cattle faeces as inocula. *In vitro* dry matter digestibility values, found with the 3% faecal suspension were not different ($p>0,05$) from rumen fluid values. Omed *et al*. (1989) found that a 60 g/l faeces-artificial saliva suspension produced a more active inoculum. Bacterial separation is presumably more efficient when a higher buffer-to-faeces ratio is used. Larger concentrations of faeces (up to 120g/l of buffer) were found to result in increased *in vitro* digestion and/or rates of gas production (Akhter *et al*., 1994; Solangi, 1997, as cited by Omed *et al*., 2000). Dilutions of dry matter between 1:20 and 1:50 produced similar gas volumes. Above or below this, gas production from fibrous substrates was depressed (Aiple *et al.,* 1992). A trade-off appears to exist between the mass of faeces used and the potency of the inoculum. Too small a mass of faeces contains insufficient cellulolytic

bacteria, while too large a mass of faeces inhibits separation of bacteria (Solangi, 1997, as cited by Omed *et al*., 2000).

#### *4.2.1.7 Addition of nitrogen supplement*

El-Shaer *et al.* (1987) found that the inclusion of urea in the buffer improved correlation with *in vivo* digestibility for all assays, involving grasses. Nsahlai & Umunna (1996) replaced urea with ammonium sulphate and found this to be equally successful.

#### *4.2.1.8 Mixing of the sample and faeces liquor*

It is necessary that the faeces liquor be mixed intimately with the forage sample. Samples are normally shaken for 30 seconds to suspend the solids, after adding faeces liquor and sealing the digestion tubes. Following the initial suspension, three different forms of agitation have been used: a shaking water bath (El Shaer *et al*., 1987), a rotor contained within an incubator (Gonçalves & Borba, 1996) or manual shaking at regular intervals (Omed *et al*., 1989). All aim to ensure that the intimate mixing, between inoculum and substrate continue (Omed *et al*., 2000). It is possible that periodical suspension allows bacteria to settle and colonise virgin substrate, whereas constant mixing prevents this (Omed *et al*., 2000).

#### *4.2.1.9 Duration of incubation*

El Shaer *et al.* (1987) showed that a 48h incubation in faecal liquor was sufficient for most forages. However, for highly digestible forages, accurate predictions of digestibility could be obtained with a reduced incubation period of 36h (Omed *et al.,* 1989), while a 72h incubation was needed to provide a good correlation with *in vivo* data for straws (El Shaer *et al.,* 1987).

### 4.2.2 Use of faecal fluid inoculum for gas production

There have been quite a few studies, using inocula from a suspension of sheep faeces, in the *in vitro* gas production technique.

Mauricio *et al*. (2001) demonstrated that using faeces liquor was associated with a longer lag phase. This is likely to be due to the fact that the faecal micro-organisms, originated mainly in the caecum/colon, where fermentation

activity is lower than in the rumen. The lag phase is the period when the micro-organisms present develop, to enable digestion of the substrate. The length of the lag time is longer with faeces liquor than with rumen liquor, because the micro-organism population is lower and they are in a 'state of suspended animation' from which it takes a longer period of time to begin growing and dividing. A number of environmental factors, in this instance being low substrate availability and the higher oxygen concentration, bring this about. Thus, micro-organisms in faeces are likely to exists in a survival mode' with low metabolic activity, rather than in an actively growing and dividing state. It can be concluded that faecal matter has potential as an alternative inoculum to rumen liquor for the *in vitro* gas production technique, but methods of overcoming the longer lag phase with faeces require further research. El-Meadaway *et al*. (1998) found that total gas production values were similar, regardless of the source of inoculum.

Lowman *et al*. (1999) suggested that although faecal material can be a suitable alternative to rumen liquor, separate calibrations with *in vivo* data are required as the rumen digesta and faecal inoculum produced slightly different gas production profiles. This agrees with the results of Harris *et al*. (1995). The different profiles may be due to the different bacterial populations in each inoculum (El-Meadaway *et al*., 1998).

### 4.2.3 Microbial population

Evidence suggests that most rumen organisms fail to survive into the large intestine, but it is likely that the bacterial community is self-sustaining. The bacterial population of the large intestine and faeces of ruminants includes numerous bacterial species present in the rumen (Omed *et al*., 2000). The time in the large intestine permits multiplication, so species depleted by proteolytic activity have a chance to recover, provided niches are available. The hindgut of the faeces donor needs slowly fermentable nutrients to stimulate multiplication of cellulytic digesters (Aiple *et al*., 1992).

Gonçalves & Borba (1996) reported incidences of lower *in vitro* digestibilities using faecal liquor and concluded that this could be due to differences in the microflora present. It is unlikely that lower bacterial numbers in the large

intestine are responsible, as the bacterial density there is similar to that in the rumen ($10^{10}$-$10^{11}g^{-1}$ ) (Allison, 1984, as cited by Omed *et al*., 2000). This suggests that the differences can be due to fewer cellulolytic species being extracted into the liquor. It may be compounded by the supply of nutrients obtained from the rumen fluid. Faecal fluid may lack trace elements and micronutrients, reinforcing the effect of forage deficiencies on digestibility. Few soluble carbohydrates will be available, consequently non-cellulolytic microbial growth will be inhibited until solubilisation occurs under the actions of the cellulolytic microbial population (Omed *et al*., 2000). El-Meadaway *et al*. (1998) concluded that the relative size of the total and cellulytic populations, were higher for rumen fluid than for faecal fluid with a greater number of bacterial genera being isolated from rumen fluid.

The difference in rate of gas production between rumen liquor and faeces as sources of micro-organisms, can be explained by the diversity of types of micro-organisms present. The diversity is less in the faeces and thus less efficient digestion will result in a slower rate of gas production (Mauricio *et al*., 2001).

Although the hindgut of ruminants is regarded more as a fermentation chamber for structural carbohydrates escaping the rumen, it can be seen that the microbes present are very adaptable and able to degrade non-structural carbohydrates as well (Aiple *et al*., 1992).

### 4.2.4 Factors affecting the second stage incubation

Centrifugation of faecal liquor at 2000 *g* for 20 minutes was recommended by El Shaer *et al*. (1987), but O'Donovan (1995), as cited by Omed *et al*. (2000) encountered problems with centrifugation, for upon completion, some of the forage samples failed to settle, requiring supernatant removal by pipette.
Filtration through sintered glass has also been reported to be problematic (El Shaer *et al*., 1987). The acid pepsin treatment, used by El Shaer *et al*. (1987), was copied from Tilley & Terry (1963), but it was recommended that the

concentration of pepsin be raised from 2 g/l to 4 g/l, as 2 g/l produced lower than expected digestibilities.

### 4.3 THE EVALUATION OF THE ENZYME TECHNIQUE

Although methods using microbial inocula have been widely and successfully used for a range of different forages, there are inherent problems in their use. Attention has therefore been focused more recently on the replacement of microbial inoculum methods by enzyme techniques, which use crude cellulase preparations (Jones & Theodorou, 2000).

Enzymatic techniques could have the advantage of being completely independent of the animal, which should result in less variation, thereby making this technique relatively simple to standardise. Conversely, the biological validity of the results can be limiting, as a result of incomplete enzymatic activity compared with the ruminal environment (Stern *et al.,* 1997).

Cellulytic enzymes as alternatives to rumen fluid are clearly an attractive prospect if their use gives results comparable to those obtained with inoculum methods. The use of enzymes eliminates the need cannulated animals and anaerobic procedures, thereby simplifying analytical methodology and eliminating the variability in activity between analytical batches (Jones & Theodorou, 2000).

The use of enzymes is based on the chemical constituents of forages, which can be divided into those making up the structure of the plant (cell wall constituents) and those contained within the cell (cell contents). The cell contents are essentially completely digestible *in vivo* and comprise soluble carbohydrates, starch (in some forages), protein, organic acids, lipids and soluble minerals. The cell wall constituents (essentially cellulose, hemicellulose and lignin) vary in digestibility depending on their polymeric configuration, degree of crystallinity and degree of lignification. Lignin itself is largely indigestible under anaerobic conditions and its presence exerts a profound effect on the digestibility of the cell wall fraction associated with it. The *in vivo* digestibility of the cellulose and hemicellulose therefore varies

from completely digested when unlignified, to completely undigested, when highly lignified. The cuticle and surface waxes are also indigestible to a large extent (Jones & Theodorou, 2000).

The enzyme activities sought for analytical procedures to predict *in vivo* digestibility need to reflect the digestive processes in the ruminant. The procedure needs to remove the soluble constituents (cell contents) and to solubilise unlignified and moderately lignified cell wall to a significant extent. The extent of solubilisation does not necessarily need to be as complete as in the *in vivo* technique, but needs to reflect the effects of lignification in a comparable and correlated way. Soluble constituents are readily removed in the pre-treatment with acid pepsin or neutral detergent, which form part of most of the proposed enzyme methods. The presence of starch in certain forage crops (maize, cereals) needs to be taken into account, since it is not removed by acid pepsin, neutral detergent or incubation with cell wall degrading enzymes. An additional treatment with amyloglucosidase or mild acid hydrolysis is therefore required in the analysis of forages or feeds containing starch (Jones & Theodorou, 2000).

The cell wall degrading enzyme used must be sufficiently active to achieve the second objective, namely the solubilisation of a significant amount of the cell wall. Crude cellulase preparations are readily available in liquid or solid form, all of which appear to hydrolyse both cellulose and hemicellulose. There is a wide disparity in the activity of cellulases from different fungal sources. It is thus essential to establish the activity of the cellulase by reference to plant cell wall material or cellulose paper and not to soluble derivatives, such as carboxymethyl-cellulose. Crude cellulases from *Trichoderma* species have generally been found to be the most reliable sources of crude cellulase, but the activity of enzymes of different suppliers should always be checked before use (Jones & Theodorou, 2000).

### 4.3.1 Sources of enzymes

Fungi and other micro-organisms, which produce enzymes able to digest plant fibre, are very widely distributed in nature, although those able to

degrade crystalline cellulose are less common. Micro-organisms in the gut of herbivores enable these animals to survive on diets largely composed of plant fibre and therefore vast areas of natural and improved grasslands are thereby utilised for the production of food (Jones & Theodorou, 2000).

Ideally, the enzyme system used to predict *in vivo* digestibility would be derived from rumen micro-organisms, but these have been unsuccessful in comparison with aerobic fungi and generally gave poor results. This is related to the anaerobic growth habit of micro-organisms in the rumen, which means that the yield of extracellular hydrolases is poor in comparison with that of their aerobic counterparts. Other factors in enzyme production include the fact that rumen bacterial cellulases are not extracellular but are often produced in a large complex, the cellulosome, which is tightly associated with the bacterial cell or bound to the substrate that is being degraded (Jones & Theodorou, 2000).

In view of the above mentioned difficulties, attention has been directed towards the use of aerobic cellulolytic fungi. The cellulase and associated enzyme systems of these fungi are extracellular and are, therefore, readily recovered from the culture fluid in which the organism is grown. Culture solutions comprise a mixture of salts, nutrients and cellulose at the requisite pH. After a growth period of 7-10 days under aerobic conditions, the culture fluid is removed by filtration and freeze-dried or an acetone powder is prepared (Jones & Theodorou, 2000).

*Trichoderma* cellulases have generally been found to be more active than those of other fungi, when using native cellulose or plant cell walls as substrates. It is evident that the complex comprises several enzymes necessary for attack of highly orientated forms of cellulose. This includes exocellulase and endocellulase, which attack amorphous regions of the cellulose polymer, producing shorter chain oligomers, and a cellobiohydrolase, which hydrolyses cellobiose to glucose. It is essential that cellulases used for forage and feed analyses retain all the components of the complex, otherwise poor activity is obtained (Jones & Theodorou, 2000).

### 4.3.2. Different pre-treatments on cellulase digestion

#### *4.3.2.1 Incubation with acid pepsin*

Incubation with pepsin in 0.1 mol/l hydrochloric acid prior to cellulase treatment was shown, by Jones & Hayward (1975), to increase the amount of dry matter solubilised and to significantly improve the correlation of enzyme solubility with the *in vivo* digestibility of grasses. These authors concluded that acid-pepsin treatment improved the accessibility of the cell wall polysaccharides to the cell wall degrading enzymes.

Pepsin-cellulase solubility was also found to be highly correlated with *in vitro* (inoculum) digestibility for grasses and clover, with very similar regression equations derived from different species. This method was found to be highly reproducible (Jones & Theodorou, 2000).

The only modifications to the original method described by Jones & Hayward (1975) are (1) the reduction of incubation time in cellulase from 48h to 24h and (2) the use of a liquid *Trichoderma* cellulase.

It was concluded that the enzyme method could accurately predict *in vivo* digestibility, provided samples of known *in vivo* digestibility similar to those being tested were included as standards in each run (Jones & Theodorou, 2000).

The enzyme solubility was less accurate than the inoculum method for predicting the digestibility of the legumes, when Terry *et al*. (1978), as cited by Jones & Theodorou (2000), compared the pepsin-cellulase technique with the two stage inoculum method (Tilley & Terry, 1963). For the inoculum method, a single regression equation was permissible for both grasses and legumes. Separate regressions were however required for grasses and legumes, when the enzyme technique was used.

Aufrere & Michalet-Doreau (1988) have suggested an interesting variation of the pepsin-cellulase technique to cater for feeds containing starch. The method involves the following three stages. After the initial acid-pepsin incubation at 40ºC, the temperature is raised to 80ºC for 30 minutes to hydrolyse starch. The final stage is incubation with cellulase after removal of the acid pepsin. The results were highly correlated with *in vivo* digestibility for a range of forage, straw and mixed diets, containing varying preparations of

concentrates and byproducts. De Boever *et al*. (1988) have described a similar procedure with a longer heating time at 80ºC.

#### *4.3.2.2. Neutral detergent extraction*

Neutral detergent extraction, under reflux or at 100ºC, has been widely used as a pretreatment before incubation with cellulase. Jones & Bailey (1974), as cited by Jones & Theodorou (2000) showed cellulase digestion with neutral-detergent residues from grasses to be highly correlated with the voluntary intake of grasses by sheep. Improving both digestibility and intake by breeding programmes could benefit from this procedure.

Downman & Collins (1982) compared cellulase solubility, using varying concentrations of cellulase, after either pepsin or neutral-detergent pretreatment, with *in vitro* (inoculum) and *in vivo* digestibility for a range of forages, silages and complete diets. An additional digestion with amyloglucosidase was included in the enzyme methods for materials containing starch. The results indicated that the enzyme techniques were, at least, as precise as *in vitro* inoculum for predicting the *in vivo* digestibility of most forages. There were little differences between the two enzyme methods, although the pepsin-cellulase method was easier to manipulate and less time consuming than the detergent method. An advantage of the latter was that samples could be analyzed in fewer working days. It was concluded that both cellulase methods could be regarded as acceptable alternatives to the *in vitro* inoculum method for the routine evaluation of forages.

Downman (1993) has proposed a modified detergent cellulase method to accommodate the galactomannans found in palm kernel oil. These polysaccharides are not solubilised by cellulase, but can be solubilised by the inclusion of an additional carbohydrase, gamanase. This can be included in the buffered cellulase solution and at to increase the digestion temperature to a 40h period. This modified method significantly improved the correlation with *in vivo* metabolisable energy values.

McLeod & Minson (1982) compared the accuracy of the detergent and pepsin cellulase methods for predicting the *in vivo* digestibility of a range of grass and

legumes. Both cellulase methods were highly correlated with *in vivo* digestibility values, although pepsin pretreatment appeared to be more precise for grasses and detergent pretreatment for legumes. It was concluded that the pretreatment with detergent increased analytical error and that the method failed to produce an overall improvement in the accuracy of predicting *in vivo* digestibility; any advantage gained from shorter analysis time was offset by an increase in analytical error.

Similarly, Coelho *et al.* (1988) found no advantage of detergent, compared with pepsin, pretreatment for predicting *in vivo* digestibility for lucerne, a range of grasses and rye-lucerne mixtures. Givens *et al*. (1990a), as cited by Jones & Theodorou (2000), compared the accuracy of different laboratory techniques, in predicting the *in vivo* digestibility. These researchers concluded that the detergent-cellulase and pepsin-cellulase methods were less affected by factors, such as year of harvest, location etc. and better related to *in vivo* values than *in vitro* (inoculum) or fibre estimations.

Bughrara & Sleeper (1986) concluded that the one stage cellulase technique would be adequate as a rapid, low cost, method for screening in plant breeding programmes. More precise estimations required pretreatment with detergent or pepsin, although both enzyme methods were considered to be more rapid and convenient, and less costly, methods of predicting *in vivo* digestibility than the two stage *in vitro* inoculum method of Tilley & Terry (1963).

#### *4.3.2.3. Hot-acid extraction*

More drastic pretreatment than pepsin or neutral detergent has been proposed in efforts to increase the amount of dry matter solubilised by cellulase by levels comparable with *in vivo* digestion. Krichgessner & Kellner (1977), as cited by Jones & Theodorou (2000), found refluxing with 2 mol/l hydrochloric acid for 30 minutes, followed by digestion with cellulase and then pepsin, to give values numerically similar to *in vivo* digestion for a range of forages.

Pace *et al*. (1984) found *in vitro* (inoculum) values for grasses and legumes to be highly correlated with pepsin-cellulase solubility and the reflux acid method. Bughara *et al*. (1989) found that the reflux acid method compared

favorably with the detergent and pepsin methods when applied to lucerne forages.

De Boever *et al*. (1988) compared the accuracy of different enzyme techniques and *in vitro* inoculum for predicting the *in vivo* digestibility of maize silage, grass silage and grass hay. The enzyme techniques included: (1) the 2 mol/l acid-cellulase procedure (2) pepsin-cellulase solubility with varying incubation times for the pepsin and cellulase stages and (3) a variant of the pepsin-cellulase method involving three stages, namely: incubation in acid-pepsin, heating the acid pepsin solution at 80ºC for 45 minutes, to remove starch and finally incubation with cellulase. The detergent-cellulase method was not evaluated, as this method had proved more difficult to manipulate than pepsin-cellulase, resulting in a lower repeatability. The *in vitro* inoculum method was the best predictor of *in vivo* digestibility for grass silage and hay, but the enzyme methods were generally superior for maize silage, particularly methods involving a hot acid pretreatment. The drastic 2 mol/l acid gave poor results, since the pretreatment remove hemicelluloses, which is generally shown to be the least digestible fraction of the cell-wall polysaccharides, in *in vivo* studies. However, the advantage of this method lies with removing starch without an additional amyloglucosidase, which is not the case with the other two pretreatment methods. Aufrere & Michalet-Doreau (1988) proposed a similar method as De Boever *et al*. (1988), but with a shorter heating time at 80°C. The 2 mol/l acid pretreatment, has sometimes given poor results, because this removes hemicelluloses, generally shown in *in vivo* studies, to be the least digestible fraction of the cell wall polysaccharides. It did, however, have the advantage of removing starch.

## 4.4 THE EVALUATION OF THE *IN VITRO* GAS PRODUCTION TECHNIQUE

There is a need for a simple, cheap and reliable laboratory technique to evaluate the nutritive value of forages for ruminant animals. Not only are the cost and animal welfare considerations making the use of animals less desirable, but also the increasing human population and demand for animal

products create a need for new animal feedstuffs and improved varieties of traditional ones (Williams, 2000). Measuring gas production during fermentation is non-destructive, cheap, rapid and can be largely automated (Pienaar, 1994).

While by no means a new concept, the measurement of gas production to evaluate forages has come to prominence in the last 10 years (Williams, 2000).

***4.4.1.1.Early techniques:*** Ouin (1943), as cited by Williams (2000), used gas tight flasks and measured gas manometrically. Measurement of gas by using liquid displacing systems was suggested in the 1960's (Johnson 1963, as cited by Williams, 2000). Later, gas production was measured by use of a syringe system to simplify measurement of gas by manometry (El-Shazly & Hungate, 1965, as cited by Williams, 2000).

Waghorn & Stafford (1993), as cited by Getachew *et al*. (1998), used a manometric method to measure gas production. Each flask was connected to a manometric measuring device, which enabled gas volumes to be measured at atmospheric pressure. The applicability of this method seems to be limited in routine feed evaluation, because it can handle only a small number of samples. The accuracy of the manometric, or glass syringe, systems depends on the accuracy of reading the gas volume in the syringes and reading the manometer, which may be low. Both systems require a periodic reading of the gas volume produced, which makes the measurements laborious and time-consuming (Cone *et al*., 1996).

***4.4.1.2. The Hohenheim gas test:*** This fermentation process was conducted within large glass syringes, so that the gas produced pushed the plunger outwards and the total volume produced was recorded after 24h (Menke & Ehrensvard, 1974, as cited by Williams, 2000). When larger samples are incubated, more gas is produced and this necessitates frequent pushback of the plunger which is inconvenient and is also a potential source of error. The syringes are also narrow and it is difficult to place a large amount (>500 mg)

of sample, particularly those that are bulky in nature e.g., straws and stovers (Getachew *et al*., 1998). This evaluation system combines the total gas volume after 24h with the concentration of crude protein (CP), crude fat, crude fibre and ash of the feed to predict metabolisable energy (ME) (Williams, 2000).

Blümmel & Ørskov (1993) have adapted the Hohenheim gas test by recording the change in gas production in the glass syringes at regular time intervals, so that a measure of the kinetics of fermentation is obtained, as well as the end-point value. A thermostatically controlled water bath was used.

The main advantages of the modified method (Blümmel & Ørskov, 1993) are that there is only a minimal drop in temperature of the medium during the period of recording gas readings on incubation of syringes in a water bath. This is particularly useful for studying the kinetics of fermentation where gas volumes must be recorded at various time intervals. Secondly, an increase in the amount of sample reduces the inherent error associated with gravimetric determination needed to determine concomitant *in vitro* apparent and true digestibility (Blümmel *et al*., 1997).

***4.4.1.3. The manual transducer technique:*** Theodorou *et al*. (1991), as cited by Williams (2000), used a pressure transducer to measure headspace gases in sealed serum bottles. The use of serum bottles isn't as expensive as the glass syringes used by Menke *et al*. (1979). The pressure and volume were recorded at regular intervals of approximately every 3-4h for the first 24h, and then less frequently to the end of fermentation at about 120 or 144 hours. The headspace gas is removed after each reading. The cumulative gas profile represents the kinetics of the fermentation process of individual feedstuffs.

Only one pressure transducer is required and a larger number of samples can be handled at a time and at lower cost. However, the large number of ingredients used complicates medium preparation and the method also involves an aseptic technique, which makes initial preparation cumbersome.

The accumulated pressure is not released and this may negatively affect microbial fermentation. There may also be a change in the solubility of the gases in the medium, which may cause error in gas measurement (Getachew *et al.,* 1998). Periodic reading of a digital display and the manual release of overpressure are still, however, needed, making the system less suitable for routine analysis (Cone *et al*., 1996).

***4.4.1.4. The automated systems***: Mauricio *et al*. (1999) described the Reading Pressure Technique as an *in vitro* feed evaluation system, based on a semi-automated gas production technique. The simplicity, low cost and high capacity of this system makes it ideal for situations where either budget constraints or the level of technical expertise required render the more complex systems inappropriate.

Beuvink *et al*. (1992), as cited by Williams (2000), used a closed system, based on the weight of the fluid that is displaced by fermentation gas for 24 individual bottles. The change in weight was recorded and a calculation made to convert the weight to gas volume, which was then registered by a data logger.

This system needs to be equilibrated, before the actual measurement starts. This is laborious and complicated (Getachew *et al*., 1998). It is also technically difficult to maintain and has a relatively high internal resistance, introducing an artificial lag time (Cone *et al*., 1996).

Pell & Schofield (1993) first described the use of computerised pressure sensors to monitor gas production. Several variables were investigated, such as sample size, inoculum size, vessel size and type of pressure sensor. Ranges, within which gas production could be accurately measured, were then reported. A revised procedure by Schofield & Pell (1995) added a new step, which consisted of recalibration of the pressure sensors by infusing a known volume of $CO_2$ every time they were used.

An automated version of the manual transducer technique (called the

automated pressure evaluation system (APES)), was first reported by Davies *et al*. (1995), as cited by Williams (2000). The system allowed for 50 gas-tight culture bottles, each of which was fitted with a pressure sensor and solenoid valve linked to a computer for continuous monitoring of all bottles. During fermentation, each solenoid valve opens to release the accumulated gas when the pressure sensor registers a pre-set gas pressure. The number of vents and the time between each vent for each bottle are recorded automatically by a computer and are then plotted to give cumulative gas profiles.

Cone *et al*. (1996) described a fully automated time related gas production apparatus. It uses a combination of pressure transducer and an electric valve for every bottle. Each valve opening represented a fixed amount of gas and the fermentation kinetics were determined by recording the time of each valve opening on a data logger (Williams, 2000). Frequent recording of gas production, offers an advantage over manual recording of gas volume (Getachew *et al*., 1998). The fully automated system has a high initial cost, complex and high maintenance, but is not so labour intensive (Mauricio *et al*., 1999).

The general principle of what the different techniques measure is the same. The technique is similar to the other *in vitro* digestibility procedures, which use milled substrates, an anaerobic medium and an inoculum of a mixed microbial population from the rumen. The pre-weighed substrate is suspended in a medium, the mixture warmed to ~39°C and a freshly collected sample of rumen fluid added as inoculum. From that moment, the production of gas resulting from fermentation is recorded, either at the end of fermentation or at regular time intervals, for those methods that measure fermentation kinetics. A cumulative profile is plotted from the results at the end of fermentation. The curve records the production of a waste product by the micro-organisms, and so reflects the kinetics of microbial activity (Williams, 2000).

*In vitro* gas production is related solely to fermentation in the rumen, rather than to digestibility in the whole digestive tract which also includes enzymatic

digestion, absorption and hindgut fermentation.

Rumen fermentation is mainly the result of the activity of the bacteria, protozoa and fungi in the rumen (Williams, 2000).

### 4.4.2. Origin of gas

When a feedstuff is incubated with buffered rumen fluid *in vitro*, the carbohydrates are fermented to short chain fatty acids (SCFA), gases (mainly $CO_2$ and $CH_4$) and microbial cells. When substrate is fermented, acetate, propionate and butyrate are produced (Getachew *et al.,* 1998). The gas produced in the gas technique is the direct gas produced as a result of fermentation ($CO_2$ and $CH_4$) and the indirect gas produced from the buffering of SCFA ($CO_2$ released from the bicarbonate buffer) (Getachew *et al.,* 1998).

### 4.4.3. Factors that have an influence on the gas production technique

As with the Tilley &Terry (1963) *in vitro* technique, Cone *et al*. (1996) found that the shape of the gas production curve was influenced by several factors. The chemical composition of the substrate, the ration and the adaptation of the donor animal to the ration, the time of obtaining the rumen fluid from the donor animal and the concentration of the substrate*.*

#### *4.4.3.1. Sample characteristics*

The preparation of a forage sample is fraught with many factors that should be considered with the *in vitro* gas production technique. It is generally recognised that a smaller particle size increases the surface area available for microbial degradation (Williams, 2000). Consequently, most authors, mill the sample through a 1mm screen, for routine gas production studies (Menke *et al*., 1979; Pell & Schofield, 1993; Theodorou *et al*., 1994)

The method and temperature of drying the samples, used by most groups, is either freeze-drying or oven-drying at low temperatures, either 60°C or 70°C (Beuvink *et al*., 1992; Pell & Schofield, 1993). Oven drying of the samples eliminates the volatile constituents from fermented substrates, thus reducing the indirect gas produced from their reaction with the buffer (Deaville &

Givens, 1998). When Cone *et al*. (1994), as cited by Williams (2000), compared different drying conditions, it was concluded that freeze-drying was better than oven drying. An increase in gas production was found, when Sanderson *et al*. (1997) freeze-dried and milled the samples compared to chopped or unchopped fresh forage.

#### *4.4.3.2 Inoculum characteristics*

Another factor which can influence the *in vitro* gas production profiles, is the microbial activity of the inoculum (Jessop & Herrero, 1998, as cited by Nagadi *et al*., 2000b). This could be affected by the frequency of sampling of ruminal liquor (Nagadi *et al*., 1999, as cited by Nagadi *et al*., 2000b), the time of collection (Pell & Schofield, 1993; Cone *et al.,* 1996), animal species (Williams, 2000), the extent of dilution with buffer and the preparation of the inoculum (Pell & Schofield, 1993). Combining rumen fluid from different animals will minimise the difference in activity of the inoculum (Cone *et al*., 1996).

Therefore, differences in the activity of micro-organisms from rumens of different species or of the same species, but different diets, results in the necessity to describe the conditions of the donor animal, when conducting *in vitro* gas production evaluations (Williams, 2000).

#### *4.4.3.3. Diet*

The diet of the host animal influences the chemical environment within the rumen and subsequently the microbial population of ruminal fluid (Weiss, 1994, as cited by Nagadi *et al*., 2000b). Several studies have indicated that the diet of donor animal influences, both the total gas production (Menke & Steingass, 1988, as cited by Nagadi *et al*., 2000b) and the gas production profiles (Cone *et al.,* 1996). Pell & Schofield (1993) and Theodorou *et al.* (1994) used ruminal fluid taken from donor animals fed on hay only. Others used ruminal fluid taken from host animals fed on a particular ratio of hay to concentrate, although the ratio varied between groups and the composition of the concentrate is rarely specified (Cone *et al.,* 1996). These differences in the diet of donor animals, may affect microbial activity or concentration and subsequently, the gas production profiles and, therefore, causes differences

in gas production data between laboratories (Moss *et al*., 1998, as cited by Nagadi *et al*., 2000b). Nagadi *et al.* (2000b) showed that changes in the microbial concentration in ruminal fluid caused by variation in diet, altered the gas production profile.

#### *4.4.3.4 Media composition*

The living rumen provides an ideal environment for its anaerobic micro-organisms in terms of pH, temperature, buffering capacity and nitrogen in the urea form. The micro-organisms also behave symbiotically, by providing each other with micronutrients, such as branched chain fatty acids and vitamins (Williams, 2000). Cellulytic bacteria have an optimum temperature and pH for growth and if this is not optimum, then gas production will be decreased (Russell & Dombrowski, 1980, as cited by Adesogan, 2002).

All media, used currently, contain buffers, a reducing agent, a nitrogen source, mineral and vitamins and resazurin, as a redox potential indicator. The $CO_2$ is used during the medium preparation, to ensure a low redox potential at the time of inoculation. This low redox potential is very important in fermentation of fibrous forages (Williams, 2000).

High phosphate buffers reduce gas production by utilising protons that would have been used for $CO_2$ production (Schofield, 2000, as cited by Adesogan, 2002). If the rumen fluid inoculum to buffer ratio is greater than 1:2, the blank will no longer truly represents the contribution of inoculum to gas production (Cone *et al*., 1997). Measurement of pH is to be recommended at the end of fermentation to ensure sufficient buffering capacity was available (Williams, 2000).

One of the most challenging problems associated with using gas production methods, is that the amount of gas produced varies with different molar proportions of volatile fatty acids (VFA) (Stern *et al*., 1997). Schofield & Pell (1995) suggested that it is important to monitor the molar proportion of VFA to correct for such differences.

The molar proportions of different short chain fatty acids (SCFA) (acetate, propionate and butyrate) produced are dependent on the type of substrate (Blümmel & Ørskov, 1993). Therefore, to compare gas data between different forages, it is also useful to examine differences in the acetate:propionate ratio at the end of fermentation (Groot *et al*., 1998). Rapidly fermentable carbohydrates yield relatively more propionate as compared to acetate, and the reverse takes place when slowly fermentable carbohydrates are incubated. If fermentation of feeds leads to a higher proportion of acetate, there will be a concomitant increase in gas production compared with a feed with a higher proportion of propionate. In other words, a shift in the proportion of SCFA will be reflected by changes in gas production (Getachew *et al.,* 1998).

Gizzi *et al.* (1998) found that the higher values for acetate, propionate and butyrate measured in the fermenters after 24h is likely to be a consequence of the accumulation of end products in the closed system.

Protein fermentation influenced gas production mainly in the initial hours of incubation, because the major part of protein is part of the soluble fraction (Cone *et al*., 1999). Gonzàlez-Ronquillo *et al*. (1998) found that measuring gas production to estimate microbial fermentation, is a useful technique but high differences in protein content may bias the results.

Caution in the interpretation of gas production profiles, particularly for forages of heterogeneous composition, should also be kept in mind. When the cell wall and cell contents predicted gas production profiles were compared to the measured profiles, there was generally an overestimation. These results suggest that gas production profiles are not necessarily linearly related to degradation or fermentation of substrates. For forage evaluation it is important to distinguish between degradation and fermentation, as not all degraded cell wall material is necessarily fermentable (Groot *et al*., 1998).

### 4.4.4 Some correlations with other techniques

Comparing *in vitro* techniques with each other is pointless, unless they are

being related to the same set of *in vivo* data and one can determine which technique is a better predictor of the *in vivo* parameters (Williams, 2000).

Menke *et al*. (1979) found that the *in vitro* gas measurement correlated well with the *in vivo* organic matter (OM) digestibility. Close correlation between gas production and *in vivo* digestibility was also reported by Menke & Steingass (1988), as cited by Williams (2000).

Prediction of metabolisable energy is more accurate when based on gas measurements (Getachew *et al*., 1998). Aiple *et al*. (1996), as cited by Getachew *et al*. (1998), found that for predicting nett energy content of individual feeds, the gas method was superior to the enzymatic and crude nutrient technique. Pell & Schofield (1993) suggested that under conditions in which nutrients are not limiting, gas production is a direct measure of microbial growth, and in some respects is a better index for predicting forage ME than the indirect measure based on NDF disappearance. Nataraja *et al*. (1998) concluded that the use of the *in vitro* gas production technique has the potential to be applied to determine ME.

High correlations between gas production and NDF disappearance, $r^2$ = .99 (Pell & Schofield, 1993) or gas production and DM disappearance, $r^2$ = .95 (Prasad *et al.,* 1994), have been reported. However, gas data are more difficult to interpret than neutral detergent fibre disappearance, because gas is generated from a wide range of substrates, including both soluble and insoluble fibre components (Schofield *et al*., 1994).

The gas production technique and triphasic model proved to be an alternative to the Tilley & Terry (1963) technique and nylon bag technique (Cone *et al*., 1999). Cone *et al*. (1997) concluded that the cumulative gas production profiles could be divided into three phases. The first phase representing the fermentation of the soluble fraction, the second phase representing the fermentation of the non-soluble fraction and the third phase, which is not caused by fermentation of the feedstuff, is the consequence of microbial turnover which becomes detectable when substrates become exhausted.

Thus, fermentation kinetics can be determined for both the soluble and non-soluble fractions, which makes it possible to compare with the Tilley & Terry (1963) technique and nylon bag technique.

Cone *et al*. (1998) proved that the gas production technique is an accurate, reliable and cheap alternative for the *in situ* technique to determine differences in fermentation characteristics and feed evaluation parameters of different grass samples. Khazaal *et al*. (1993) also concluded that the gas test has good potential for predicting apparent digestibility and intake, to a level close to that of the nylon bag technique.

Pienaar (1994) found that the rate of fermentation determined by gas production, agreed well with the results obtained with the *in sacco* nylon bag technique. A largely unexplained discrepancy was found between the *in vivo* results and the other methods, when the latter was corrected for rate of passage (Pienaar, 1994).

Schofield & Pell (1995) used gas production to measure and determine the kinetics of the neutral detergent soluble carbohydrate fraction and found a good correlation between the volume of gas produced and the mass of fibre digested in the samples.

### 4.4.5. Advantages and disadvantages of the gas production technique

The advantages of this technique are multiple. The amount of material required ranges from 0.1 to 1 g, making it suitable for materials available in limited quantities (e.g. from plant-breeding trials, plants grown under special conditions, etc.) (Williams, 2000).

The initial equipment required could be expensive, but, as the hours for measuring are significantly reduced, it is a matter of balancing the capital costs of the equipment with the labour costs (Kitessa *et al*., 1999; Williams 2000).

The use of gas production to study carbohydrate digestion presents an advantage over the traditional gravimetric method, because it accounts for both soluble and insoluble substrates (Pell & Schofield, 1993).

Nherera *et al.,* (1999) concluded, that the *in vitro* gas production technique has the potential, to be an important tool for assessing degradation (rate and extent of fermentation over 96h) and estimating *in vivo* microbial nitrogen supply (24h gas test) of diets, supplemented with leguminous fodder tree forage. The 24h gas production test was able to reflect trends in gas production and degradation, similar to those established in the 96h gas production test. This also offered the advantage of being able to predict microbial nitrogen yield. It is, therefore, recommended that the shorter incubation be used, as this will enable more feeds to be evaluated over a short period of time.

Nagadi *et al*. (2000a) harvested ruminal microbes to determine the effect they had on gas production dynamics. These researchers found no alteration in the pattern and this increased the potential of the gas production technique, enabling more control of the nature of the microbial inoculate and microbial concentration. This demonstrates that protein-energy interactions can be studied using the *in vitro* gas production technique.

The relation between gas production and feed intake is interesting, as being of immediate practical relevance (Williams, 2000). Roughage which produced proportionally less gas per unit substrate truly degraded, had higher feed intakes (Blümmel *et al.,* 1997).

It is accurate enough to record small changes in the fermentation process. Changes from a semi aerobic, hydrogen producing type fermentation, to a strictly anaerobic type of fermentation, could cause changes in gas pressure, which are not necessarily related, to the disappearance of the solid substrate. Fortunately, these changes are small and usually only occur during the start of the fermentation process (Pienaar, 1994).

Information obtained from gas production remains an indicator of what happens in the rumen, rather than the whole animal, because it measures kinetics of fermentation (Williams, 2000). There is also still a need cannulated animals to supply rumen fluid, but far less than for *in vitro* digestibility trials. Faeces have been used as an alternative inoculum (Aiple, 1993, as cited by Williams, 2000).

The lack of uniformity in methodology, which makes comparison of results from different groups difficult, is offset against the advantages. Standardisation in terms of kind of medium (ideal buffer concentration, micronutrients and anaerobiosis), timing of inoculum collection and diet of donor animals, still needs to be refined. Other important details include: treatment of inoculum to maximise cellulolytic organism activity, use of blanks, sample preparation in terms of drying and grinding, relationship between gas, VFA and microbial production (Williams, 2000). Williams (2000) showed that atmospheric pressure determines actual gas volumes. This is often omitted, making it difficult to compare results from different laboratories. Stirring reduces $CO_2$ supersaturating, which may also cause erroneous volume/pressure readings (Pell & Schofield, 1993).

Blümmel *et al*. (1997) found that using the *in vitro* gas production technique might select against maximum microbial yield, by favouring substrates with proportionally high SCFA yield. This intrinsic disadvantage can be overcome by combining gas measurements with determination of the undegraded residue.

Rymer & Givens (1997) used three sets of apparatus and found that the results obtained, using one gas production system, cannot reliably be extrapolated to another.

### 4.4.6 Applications of the gas production technique

Differences in quality of hay, grass or silage, due to plant maturity and conditions of growth, have been investigated by Williams (2000) using the gas production technique. Gas-production kinetics is also used to rank different

species or cultivars (Piva *et al*., 1988, as cited by Williams, 2000). It has been used to determine differences in fermentability, between different chemical (Prasad *et al*., 1994) and physical pre-treatments of straw, crop residues of different cultivars or growth under different environmental conditions. The effect of addition of supplements on the fermentability of straws was also determined (Prasad *et al*., 1994).
Different browse trees, as potential feed supplement for poorly degradable roughages, have been ranked by using the gas production technique (Topps, 1992).

In the *in vitro* gas method the effects of anti-nutritive factors (ANFs) on rumen fermentation are reflected in the gas production (Getachew *et al*., 1998). Given that there are several possible mechanisms by which browse ANFs could affect microbial activity, the technique has been used to pinpoint, which mechanism is responsible for changes to fermentation (Williams, 2000). Khazaal *et al*. (1995), as cited by Nherera *et al*. (1999), reported that the gas production method was better in determining the nutritive value of fodder trees containing anti-nutritional factors.

Starch and protein are important components of forages and their fermentation can provide insight into the fermentation of the whole forage (Williams, 2000). It was reported that gas production was highly correlated with *in vitro* DM disappearance, total VFA production and starch digestion (Trei *et al*. 1970, as cited by Williams, 2000). More recently, gas production from fermentation of several starch-based substrates, proved to be an accurate index of VFA production and change in pH (Opatpatanakit *et al*. 1994, as cited by Williams, 2000).

# CHAPTER 5

## MATERIALS & METHODS OF THE DIFFERENT TECHNIQUES USED TO DETERMINE THE DIGESTIBILITY OF *A.NUMMULARIA* CV. DE KOCK

### 5.1 Experimental materials

The *A. nummularia* cv. De Kock had been harvested between the end of March and the beginning of April 2001, at the Grootfontein Research Centre in the Eastern Cape, South Africa. The stand was established in the early 1990's and had been heavily grazed by sheep, as a drought fodder, during the previous winter. It was harvested with a mechanical weed eradicator and by hand. It was sun dried and sorted into edible and non-edible material. Edible material was defined as leaf and stems with a diameter of 6 mm and less. After sorting, the material was milled through a hammermill with a 25 mm sieve size.

The crude protein (CP), ash concentration and organic matter (OM) concentration were determined according to A.O.A.C. (2000) and neutral detergent fibre (NDF) concentration according to Van Soest & Wine (1967). The following values for edible material were obtained: CP (10.29 g/kg DM), ash (14.93 g/kg DM), OM (75 g/kg DM) and NDF (60.86 g/kg DM).
Afgri, Kaalfontein silo, and Southern Associated Malsters in Alberton, South Africa supplied the maize and barley, respectively.

### 5.2. *In vivo* digestibility trial

The *in vivo* digestibility trial was conducted at the Hatfield Experimental Farm of the University of Pretoria. The experiment was carried out in a split plot design. The animals were randomly divided into two groups, so that five animals each received the different treatments within a group. The trial was run is four sequential experimental periods, each period lasting 24 days. The first 14 days of each period was an adaptation period, while the last 10 days were used for collection of data and samples. The animals were placed in metabolism crates fitted with individual feed and water troughs and were kept

under natural lightning. The crates were also equipped with urine pans for urine collection. During the experimental period, the animals had harnesses and nylon canvas bags for total faeces collection.

The basal diet consisted of *A. nummularia* cv. De Kock. In addition to the control diet of 100% *A. nummularia*, 15%, 30% and 45% of maize and barley were added to the basal diet on a dry matter basis. The diets were offered as total mixed rations, to prevent particle selection. Animals were fed a lucerne/*Atriplex* mixture (85:15) *ad lib*, when the supplemented diets were not being tested.

All the animals were used in the digestibility trial, *i.e.* five per group. The animals were weighed at the start and finish of each experimental period. Individual feed intakes were recorded daily by weighing the feed offered as well as the orts. The quantity of feed offered each day was adjusted to ensure that the feed troughs contained feed throughout the day. Samples of feed offered and orts, which were collected every morning, were pooled for proximate analysis (A.O.A.C., 2000).

All faecal output during the experimental period was recorded and a 10% sample of the daily output was stored at -10°C until the end of the collection period. At the end of the collection period, the total faeces collected for each sheep were mixed and sub-sampled. Feed and faecal samples were dried at 60°C and ground through a 1mm sieve before chemical analyses (Mahgoub *et al*., 2000).

The urine output was measured daily and collected in a container that contained 25 ml of 10% $H_2SO_4$ to prevent loss of urinary ammonia. Urine aliquots (10% of daily output) were pooled, frozen at -10°C and kept for analyses (Mahgoub *et al*., 2000).

The animals received fresh water daily on an *ad lib* basis and the remaining water was measured back and recorded before feeding commenced the next morning. Water consumption of individual animals was recorded daily and it was corrected for evaporation losses.

## 5.3. Analytical methods

### *5.3.1. Dry matter concentration*

Samples of diets and faeces were dried at 100°C for 24h in a forced drought oven in aluminium foil containers. Dry matter of milled samples was determined in porcelain crucibles. Dry matter concentration was calculated as recommended by A.O.A.C. (2000).

### *5.3.2. Neutral detergent fibre (NDF) concentration*

NDF concentration of diets and faeces samples were determined with neutral detergent solution (NDS) on a Tecator Fibertec system using the procedure described by Van Soest & Wine (1967).

### *5.3.3. Apparent digestibilities*

The apparent dry matter digestibility (DMD) for each experimental diet was calculated using the formula described by McDonald *et al*. (2002):

$$\frac{\text{Nutrient consumed} - \text{Nutrient in faeces}}{\text{Nutrient consumed}}$$

## 5.4. *In vitro* digestibility trials

*A. nummularia* cv. De Kock, was supplemented with four levels (0%,15%, 30%, 45%) of either maize or barley and was incubated *in vitro* in a buffered medium, with rumen fluid inoculum collected from sheep, as described by Tilley & Terry (1963). The two different energy sources differ in their fermentation rates. Maize is a slower fermentable and barley a more rapid fermentable energy source.

### *5.4.1. The in vitro rumen fluid technique (Tilley & Terry, 1963, as modified by Engels & Van der Merwe, 1967)*

Triplicate samples, each weighing 0.2 g, after being milled through a 1 mm sieve, were weighed into centrifuge tubes. An urea solution was made up and 2 ml added to each sample. The artificial saliva (McDougall, 1948) was prepared and placed in a water bath (39ºC) and saturated with $CO_2$. Rumen fluid was collected from rumen cannulated sheep, which have been fed good

quality lucerne hay. After filtration through cheesecloth, the rumen fluid was mixed with artificial saliva in a 1:3 ratio and 20 ml of this mixture was added to the centrifuge tubes. Before sealing the tubes with a rubber stopper, $CO_2$ was added before being incubated for 48h, while being shaken continuously. In the second stage, 20 ml of fresh pepsin and 0.1M HCl solution were added to the residue, after centrifugation at 2500 rpm (revolutions per minute) for 15 minutes. The tubes were incubated again for 48h while being shaken continuously. After 48 h the tubes were centrifuged, the residue washed with 20 ml of warm tap water and re-centrifuged. The residue was dried at 100ºC for 18 h, cooled and weighed. It was then ashed at 550ºC for 3h, to determine the organic matter (OM) mass.

### *5.4.2. The in vitro faeces fluid technique (El Shaer et al., 1987)*

Triplicate samples, each weighing 0.2 g, after being milled through a 1 mm sieve, were weighed into centrifuge tubes. Faeces from sheep, maintained on a lucerne diet, were collected within 1h of voiding and macerated, using a pestle and mortar. This was mixed in a 1:1 ratio with artificial saliva, which had previously been saturated with $CO_2$. After filtration through cheesecloth, the filtrate mixture was made up with artificial saliva to the amount needed. Together with 0.6 ml of a freshly prepared urea solution, 20 ml of the mixture was added to the tubes. The tubes were incubated, after the addition of $CO_2$ in a shaking water bath at 39ºC for 48h and then centrifuged at 2500 rpm for 15 minutes. A fresh pepsin and 0.1N HCl solution was prepared, 20 ml added to the tubes and incubated for a further 48h, being shaken to re-suspend the feedingstuff residues. At the end of the second stage, the same procedures as in technique (a) were followed.

### *5.4.3. The in vitro gas production technique (Pienaar, 1994)*

The procedure followed was an adaptation of the two-phase technique described by Tilley & Terry (1963). It involves a 72h fermentation by rumen micro-organisms in a buffer solution followed by a 48h pepsin digestion after acidification. Cumulative gas production is measured during the fermentation period.

Rumen fluid was drawn from two cannulated sheep fed *ad libitum*, one on a good quality lucerne diet, and the other on a diet of lucerne plus maize in a ratio of 5:1. The rumen fluid from the two sheep was mixed in a ratio of 1:1.

Duplicate samples, each 0.5 g, were weighed into 100 ml Scott reagent bottles. An urea solution was made up and 5 ml added to each sample. The artificial saliva (McDougall's) was prepared and placed in a water bath (39ºC) and saturated with $CO_2$. Rumen fluid was collected from cannulated sheep, which have been fed good quality lucerne hay. After filtration through cheesecloth, it was mixed with artificial saliva in a 1:2 ratio and 50 ml of this mixture was added to the centrifuge tubes, flushing the bottle at the same time with carbon dioxide. The bottle was immediately sealed and attached to the pressure sensor in the incubator.

A standard fodder sample with known digestibility, e.g. *Panicum maximum* was always included in the test run to serve as a check on the test conditions.

After 72h of incubation (with careful daily swirling) 5 ml HCl solution was added to each bottle (in three portions and mixing carefully after each addition) and 5 ml pepsin solution. The bottles were closed again and incubated for a further 48h. After 48h, the contents of each bottle were transferred to a 100 ml glass centrifuge tube. After centrifuging at 3000 rpm for 10 minutes, the supernatant was removed, the residue rinsed with hot $H_2O$ and centrifuged again. The residue was dried at 105ºC for 24h, cooled and weighed. It was then ashed at 500ºC for 6h, to determine the organic matter (OM) mass.

### *5.4.4. The in vitro cellulase technique (De Boever et al., 1986)*

To facilitate cellulolytic breakdown, the feed sample was treated with a pepsin solution. Hydrolysing at 80ºC removed the starch. Finally, cell walls were attacked by cellulase.

Duplicate samples, each weighing 0.3 g, after being milled through a 1mm sieve, were weighed into a sintered glass crucible (capacity 50 ml, porosity 1) and the bottom side was closed with a rubber stopper. A preheated pepsin-HCl (30 ml) was added and after closing the top with 'glad wrap', the crucibles were incubated (40ºC) for 24h, and stirred continuously. To hydrolyse the starch, the crucibles were placed in a warmbath (80ºC) for 45min and then

filtrated. After filtration 30 ml of the preheated cellulose buffer mixture was added and then incubated for a further 24h. The residue, after filtration and washing, was dried at 103ºC for 24h, cooled and weighed and then ashed for 1.5h at 500ºC.

### *5.4.5. The in vitro cellulase technique (Wageningen Institute of Animal Science, The Netherlands)*

Duplicate samples, each weighing, 0.3 g, after being milled through a 1mm sieve, were weighed into a sintered glass crucible (capacity 50 ml, porosity 1) and the bottom side was closed with a rubber stopper. A preheated pepsin-HCl (30 ml) was added and after closing the top with 'glad wrap', the crucibles were incubated (40ºC) for 24h, and stirred continuously. To hydrolyse the starch, the crucibles were placed in a warmbath (80ºC) for 30 minutes and then filtrated. After filtration 30 ml of the pre-heated cellulase/amyloglucosidase mixture was added and then incubated for a further 24h. The residue, after filtration and washing, was dried at 103ºC for 24h, cooled and weighed and then ashed for 2h at 500ºC.

The amyloglucosidase used was extracted from *Aspergillus niger*, (Calbiochem) while the cellulase, Onozuko R-10, was extracted from *Trichoderma viride*.

## 5.5. Statistics

An analysis of variance with the Proc GLM model (Statistical Analysis Systems, 1994) was used to determine the significance between the *in vitro* digestibility techniques and *in vivo* data. Means and standard deviations (sd) were calculated. The significance of difference (5%) between means was determined using Bonferroni's test (Samuels, 1899). Regressions for the different techniques and levels of supplementation were determined with the Proc GLM model (Statistical Analysis Systems, 1994).

# CHAPTER 6

## RESULTS AND DISCUSSION OF USING DIFFERENT TECHNIQUES TO DETERMINE THE DIGESTIBILITY OF *A. NUMMULARIA* CV. DE KOCK

The aim of this study was to determine the digestibility of *Atriplex nummularia* cv. De Kock, supplemented with three levels (15%, 30% and 45%) of either maize or barley, using different *in vitro* techniques. An *in vivo* digestibility trial was conducted, together with the *in vitro* trials.

### 6.1 The Tilley & Terry (1963) rumen fluid *in vitro* technique.

To determine how accurate the *in vitro* digestibility can be used to predict the organic matter digestibility (OMD) of *A. nummularia* cv. De Kock, supplemented with different levels of maize and barley, a comparison between the Tilley & Terry (1963) rumen fluid *in vitro* technique and the *in vivo* data is shown in Table 6.1.1 and Fig 6.1.1.

**Table 6.1.1** A comparison between the Tilley & Terry (1963) rumen fluid *in vitro* technique and *in vivo* data, to determine the predicted organic matter digestibility (OMD) of *A. nummularia* cv. De Kock, supplemented with different levels of maize and barley

| Levels | Treatment | |
|---|---|---|
| | **OMD %**<br>**Rumen fluid inoculum** | **OMD %**<br>***In vivo* technique** |
| Atriplex 0% | $39.583^{a}{}_{1}$ (±1.20) | $34.315^{b}{}_{1}$ (±10.23) |
| Atriplex+15%Maize | $65.873^{a}{}_{2}$ (±0.14) | $54.676^{b}{}_{2}$ (±7.03) |
| Atriplex+15%Barley | $62.444^{a}{}_{2}$(±10.59) | $61.283^{b}{}_{2}$ (±5.81) |
| Atriplex+30%Maize | $64.886^{a}{}_{2}$ (±2.06) | $55.692^{b}{}_{2}$ (±14.14) |
| Atriplex+30%Barley | $69.554^{a}{}_{2}$ (±0.39) | $62.448^{b}{}_{2}$ (±8.36) |
| Atriplex+45%Maize | $68.304^{a}{}_{2}$ (±0.10) | $63.954^{b}{}_{2}$ (±8.46) |
| Atriplex+45%Barley | $72.49^{a}{}_{2}$ (±10.083) | $67.033^{b}{}_{2}$ (±6.96) |

[ab] Row means with common superscripts do not differ (p>0.05)
[12] Column means with common subscripts do not differ (p>0.05)
(±) = Standard deviation

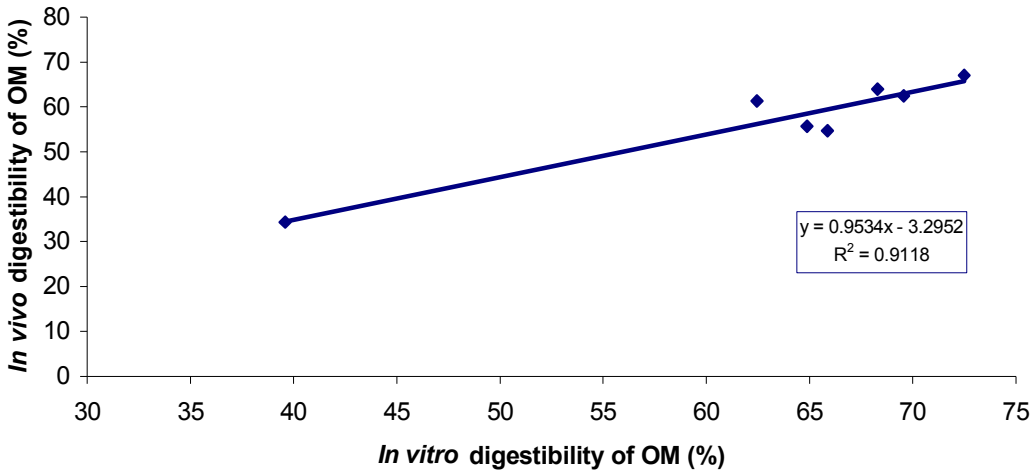


**Figure 6.1.1** A relationship between the Tilley & Terry (1963) *in vitro* technique and *in vivo* data, for the determination of a regression to predict organic matter digestibility (OMD) of *A. nummularia* cv. De Kock, supplemented with different levels of maize and barley

After *in vitro* digestibility values are determined, they are corrected, by using regression equations relating *in vitro* and *in vivo* digestibility values determined from a large sample set. (Kitessa *et al*., 1999). A regression equation usually consists of the following:

$$Y = a + bx$$

For maximum confidence in the results of a digestibility estimation, the constant ***a*** in the equation should be very small and the coefficient ***b*** should approximate to 1, indicating that digestion has reached its natural limits. If this is not found, it may be that the microbial activity in the liquor had a low activity and that more than 48 h is needed to achieve the degradation potential. With a high microbial activity, degradation of most forage will be substantially complete within the prescribed 48h, resulting in *in vitro* weight loss close to *in vivo* apparent digestibility (Omed *et al*. 2000).

It was not easy to gauge if fibre degradation was complete when the prescribed time was reached. At the cut-off time of 48h, digestion of fibre in

some forage may be incomplete and still progressing. No stable, repeatable end-point is reached. Weight losses from forages may still show significant correlation with their *in vivo* apparent digestibilities, but the data will reflect variations in their rates of digestion both within and between samples. Forages with highly digestible components may be overvalued (Omed *et al*. 2000).

The $R^2$ (coefficient of determination) of the above regression line is 0.9118, which means that 91% of the variance in *in vivo* digestibility can be explained by the *in vitro* digestibility.
A highly significant coefficient of correlation (r=0.95) was, therefore, obtained between *in vivo* and *in vitro* digestibilities. The degree of intensity of the association between the *in vivo* and *in vitro* digestibilities is therefore high.

A linear regression equation, **y =0.9534x - 3.2952**, was fitted to the data.

From the regression equation it can be seen that constant ***a*** which has the value of -3.2952 is small and the constant ***b*** with the value of 0.9534 is approaching 1, therefore the assumption is made that the digestion was fairly complete.

Several authors found that the Tilley & Terry (1963) *in vitro* technique was well correlated with the *in vivo* data. Engels & Van der Merwe (1967) found the correlation between *in vivo* and *in vitro* digestibility values of 59 South African forages to be r=0.90. The individual *in vivo* digestibilities of the forages varied between 40% and 79% and corresponded fairly closely to the *in vitro* digestibilities, with the exception of some of the grass samples. The agreement between *in vivo* and *in vitro* digestibility values of all lucerne hays was particularly good. The difference between the *in vivo* and *in vitro* OMD, especially the grasses of low digestibility, may be due to the inability of the experimental sheep to consume these grasses to the extent, which would supply maintenance needs. Khazaal *et al*. (1993) also indicated limitations with this method, in predicting *in vivo* digestibility, especially of poor quality

feeds like straws, which are usually very bulky. The difference in the *in vivo* and *in vitro* digestibility increased as herbage digestibility declined.

Kitessa *et al*. (1999) also cited some $r^2$ values from other authors, relating to the accuracy and precision of regression equations for predicting *in vivo* digestibility from *in vitro* parameters. Barber *et al*. (1984) reported 0.71 for fresh grasses, Gasa *et al*. (1989) had a value of 0.87 for by-products. Givens *et al*. (1991) reported $r^2$ values of 0.60 on straws and in Givens *et al*. (1989) a value for silage of 0.74 was recorded.

In Figure 6.1.2 and 6.1.3 a comparison is made between the different levels of maize and barley supplementation in the *in vitro* Tilley & Terry (1963) technique and the different levels of maize and barley supplementation in the *in vivo* technique.

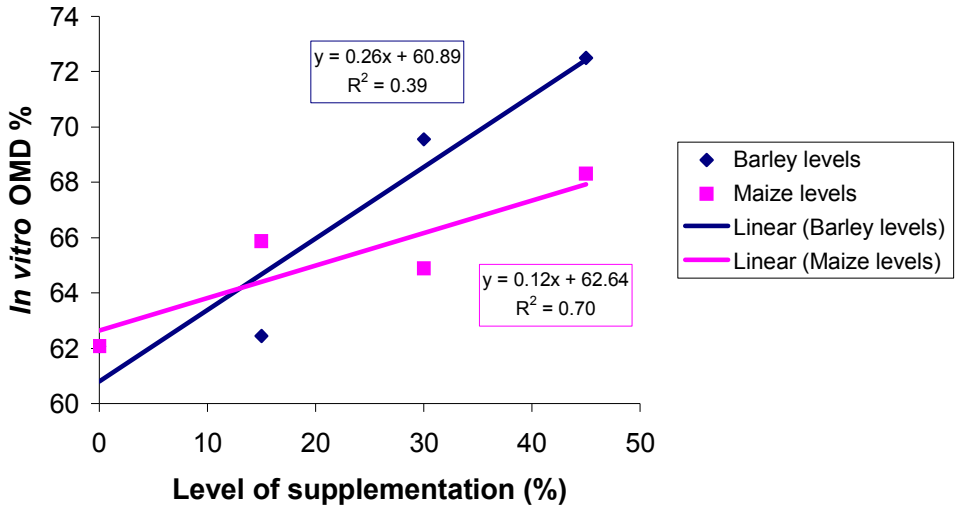


**Figure 6.1.2** A comparison between the different levels of maize and barley supplementation (%) of *A. nummularia* on the *in vitro* OMD %, with the *in vitro* Tilley & Terry (1963) technique

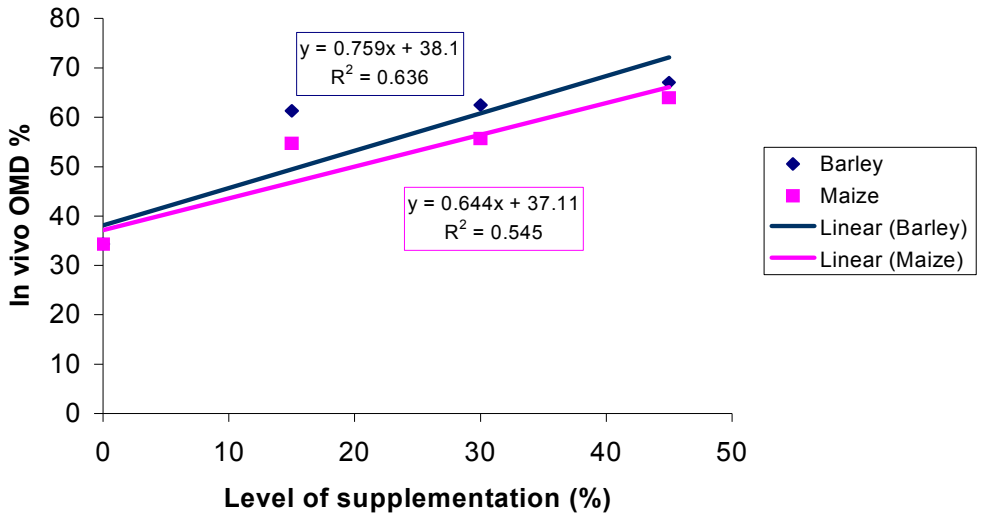


**Figure 6.1.3** A comparison between the different levels of maize and barley supplementation (%) of *A. nummularia* on the *in vivo* OMD %, with the *in vivo* technique

The differences in digestibility found between the type and level of supplementation, to *Atriplex nummularia* cv. De Kock, may be due the differences in rate of fermentation of the two carbohydrate sources. Barley is a faster fermentable carbohydrate source, while maize is slower.

Table 6.1.2 shows the mean *in vitro* OMD % compared to the mean *in vivo* OMD % over all levels of supplementation.

**Table 6.1.2** A comparison between the Tilley & Terry (1963) rumen fluid *in vitro* technique and *in vivo* data, to determine the organic matter digestibility (OMD) of *A. nummularia* cv. De Kock, over all the different levels of supplementation

| **Treatment** | **OMD %** |
|---|---|
| Rumen fluid inoculum | 66.52[a] (±1.93) |
| *In vivo* digestibility | 56.05[b] (±1.93) |

[ab] Columns means with common superscripts do not differ (p>0.05)
(±) = standard deviation

From Table 6.1.2 it can be seen that, the Tilley & Terry (1963) *in vitro* technique mean OMD % differed ($p<0.05$) significantly from the mean *in vivo* OMD %. There are several possible explanations for the difference between the *in vivo* and *in vitro* OMD %. Practical mistakes could have been made. Engels & Van der Merwe (1967) made the same suggestion, that when *in vivo* and *in vitro* processes are compared, there are obvious dissimilarities between the indigestible residues, obtained from these two types of digestion. This reflects the inherent analytical errors in the two methods.

The fermentation characteristics and microbial constitution of the rumen inocula differ between the animals used for the *in vivo* digestibility trial and the animals used for rumen inocula collection. The simulation of the rumen motility *in vitro* is also difficult. There are several factors that influence the microbial activity of the inoculum. It can vary depending on the time of collection after feeding, the diet and species of the donor animal, and animal to animal variation within species (Kitessa *et al*., 1999). It could have been that all the feed particles did not have the same exposure to the microorganisms. This may be due to forage x particle size interaction (Baumgardt & Ho, 1964, as cited by Kitessa *et al*. 1999). With *in vivo* digestibility the time of digestion is not known, and therefore, the time of rumen and gastric digestion *in vitro* could have been too long or too short.

Animals also show a considerable variation in digestive capacity, especially with certain forages, where intakes are unrestricted. (Engels & Van der Merwe, 1967).

## 6.2 The *in vitro* faeces fluid technique (El Shaer *et al*., 1987)

To determine, how accurate the *in vitro* digestibility can be used to predict the organic matter digestibility (OMD) of *A. nummularia* cv. De Kock supplemented with different levels of maize and barley, a comparison between the *in vitro* faeces fluid technique (El- Shaer *et al*., 1987) and the *in vivo* data is shown in Table 6.2.1 and Figure 6.2.1.

**Table 6.2.1** A comparison between the *in vitro* faeces fluid technique (El-Shaer *et al*., 1987) and *in vivo* data, to determine the predicted organic matter digestibility (OMD) of *A. nummularia* cv. De Kock supplemented with different levels of maize and barley

| **Levels** | **Treatment** | |
|---|---|---|
| | **OMD %**<br>**Faeces fluid inoculum** | **OMD %**<br>***In vivo* technique** |
| Atriplex 0% | $38.233_1$ [a](±0.92) | $34.315_1$ [b] (±10.23) |
| Atriplex+15%Maize | $65.152_2$ [a](±2.51) | $54.676_2$ [b](±7.03) |
| Atriplex+15%Barley | $62.300_2$ [a](±1.50) | $61.283_2$ [b](±5.81) |
| Atriplex+30%Maize | $63.159_2$ [a](±0.91) | $55.692_2$ [b](±14.14) |
| Atriplex+30%Barley | $68.053_2$ [a](±6.43) | $62.448_2$ [b](±8.36) |
| Atriplex+45%Maize | $66.253_2$ [a](±1.76) | $63.954_2$ [b](±8.46) |
| Atriplex+45%Barley | $73.3495_2$ [a](±4.70) | $67.033_2$ [b](±6.96) |

[ab] Row means with common superscripts do not differ (p>0.05)
[12] Column means with common subscripts do not differ (p>0.05)
(±) = standard deviation

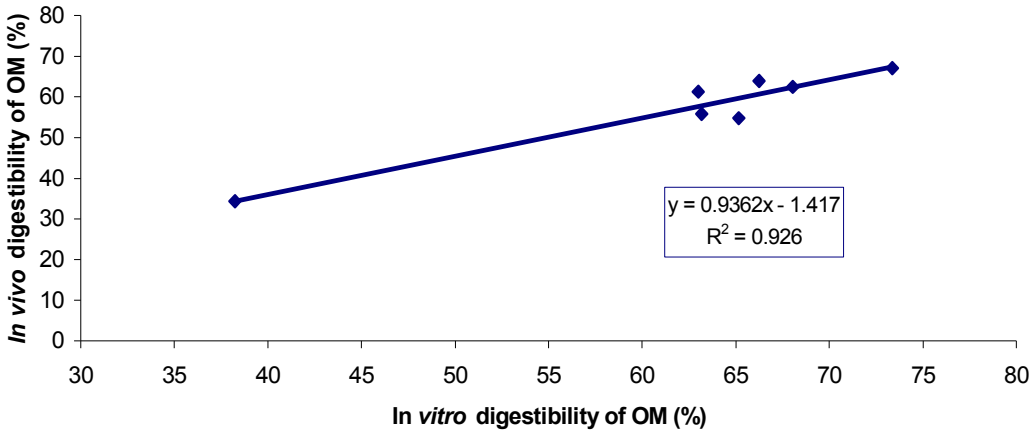


**Figure 6.2.1** A relationship between the faeces fluid *in vitro* technique (El-Shaer *et al*., 1987) and *in vivo* data, for the determination of a regression to predict organic matter digestibility (OMD) of *A. nummularia* cv. De Kock, supplemented with different levels of maize and barley

The $R^2$ of the above regression is 0.926, which means that 93% of the variance in *in vivo* digestibility can be explained by the *in vitro* digestibility.
A highly significant correlation (r=0.96) was, therefore, obtained between *in vivo* and *in vitro* digestibilities.

A linear regression equation, **y =0.9362x – 1.417**, could be fitted to the data.

From the regression equation it can be seen that the coefficient ***a***, which has the value of -1.417 is small and the constant ***b*** with the value 0.9362 is approaching 1, therefore the assumption is made that the digestion was fairly complete.

Nsahlai & Umunna (1996) also reported a strong relationship between *in vivo* digestibility and *in vitro* digestibility using inocula from a faecal suspension (r=0.88). El-Shaer *et al.* (1987) found the results correlated closely (r=0.98) with the *in* vivo digestibilities. In a study by Omed *et al*. (1989), the faeces liquor-pepsin procedure estimated *in vivo* digestibility of various feeds with precision and accuracy.

In Figure 6.2.2 and 6.2.3 a comparison is made between the different levels of maize and barley supplementation in the faeces fluid *in vitro* technique (El-Shaer *et al*. 1987) and the different levels of maize and barley supplementation in the *in vivo* technique.

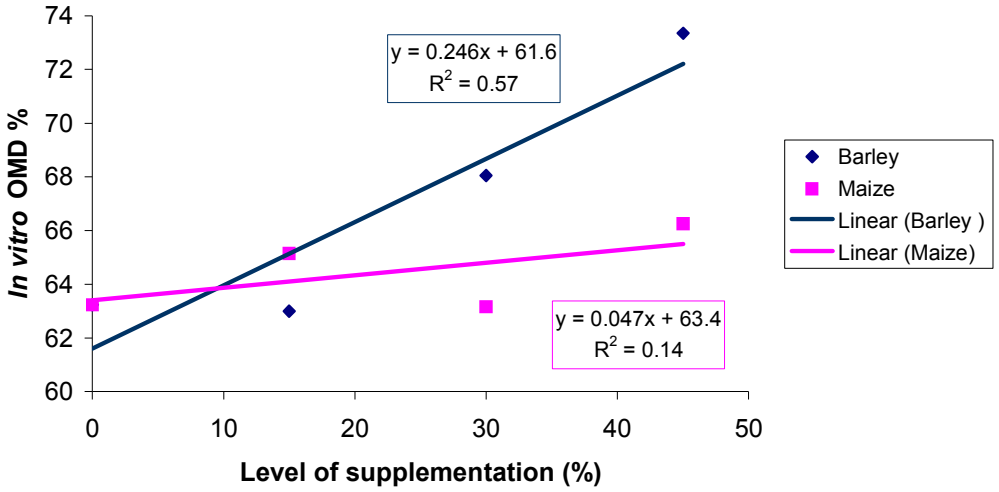


**Figure 6.2.2** A comparison between the different levels of maize and barley supplementation (%) of *A. nummularia* on the *in vitro* OMD %, with the faeces fluid *in vitro* technique

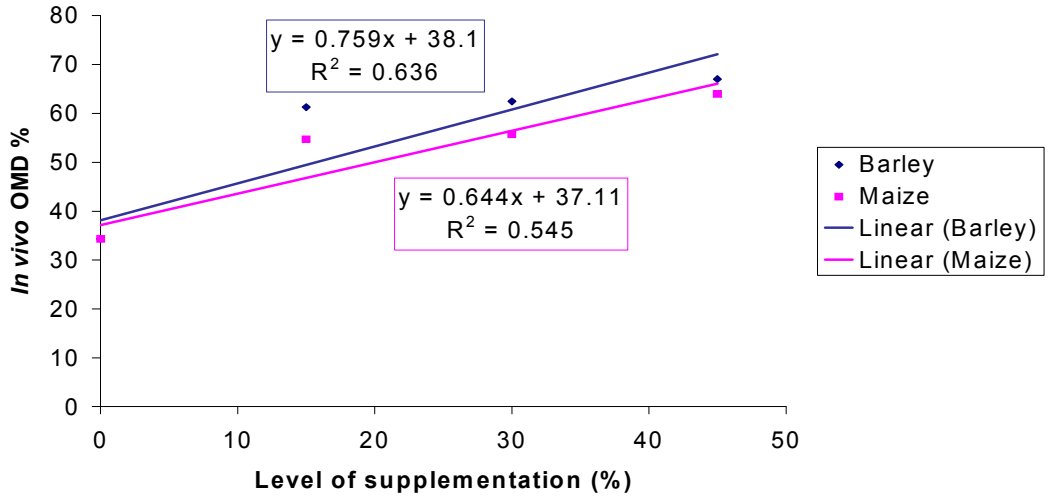


**Figure 6.2.3** A comparison between the different levels of maize and barley supplementation (%) of *A. nummularia* on the *in vivo* OMD %, with the *in vivo* technique

Table 6.2.2 shows the mean *in vitro* OMD % compared to the mean *in vivo* OMD % over all levels of supplementation

**Table 6.2.2** A comparison between the *in vitro* faeces fluid technique (El Shaer *et al*., 1987) and *in vivo* data, used to determine the organic matter digestibility (OMD) of *A. nummularia* cv. De Kock, over all the different levels of supplementation

| Treatment | OMD % |
|---|---|
| Faeces fluid inoculum | 66.03[a] (±1.93) |
| *In vivo* digestibility | 56.05[b] (±1.93) |

[ab] Column means with common superscripts do not differ (p>0.05)
(±) = standard deviation

The faeces fluid *in vitro* technique's OMD % did, however, differ significantly (p<0.05) from the *in vivo* OMD %, as demonstrated in Table 6.2.2. There are several possible explanations for this difference. As with the rumen fluid technique, practical mistakes could have been made. The fermentation characteristics and microbial constitution of the faeces inoculate, could differ from the rumen inoculate in the trial sheep, used with the *in vivo* digestibility trial. The initial microbial mass may have been lower than in rumen fluid (Nsahlai & Umunna, 1996) and the simulation of the rumen motility *in vitro* is difficult. The activity of the inoculum could also be affected by the amount of microbes present in the faecal suspension. This could be affected by the dilution of faeces with the medium (Aiple *et al*., 1992). All the feed particles may not have had the same exposure to the microorganisms, because the separation of the bacteria from the faeces may not have been as effective. The diet fed to the donor animals, from which the faeces was collected, may have also not provided enough fermentable substrate for the lower digestive tract, and therefore, microbial population in the hindgut and hence, in the faeces, was not very dense and active (Aiple *et al*., 1992).

The *in vivo* digestibility method is different from other procedures in several ways. There is an onward passage of digesta along the gut. The time of digestion is not exactly known, and the time of rumen and gastric digestion *in vitro* could, therefore, have been either too long or too short (Nsahlai &

Umunna, 1996). Aiple *et al*. (1992) studied the time of incubation of different substrates using sheep faeces. Time will have an effect on the accuracy of the laboratory method. It was observed that the initial lag phases differ between substrates, and this can lead to higher *in vitro* values, especially in the case of barley. This effect seems to depend on the nature of the starch. The same observation wasn't seen in maize. This could add to the difference between *in vitro* and *in vivo* OMD %.

### 6.3 The *in vitro* gas production technique (Pienaar, 1994)

To determine how accurate the *in vitro* gas production technique can be used to predict the organic matter digestibility (OMD) of *A. nummularia* cv. De Kock, supplemented with different levels of maize and barley, a comparison between the *in vitro* gas production data (Pienaar, 1994) and the *in vivo* data is shown in Table 6.3.1 and Figure 6.3.1.

**Table 6.3.1** A comparison between the *in vitro* gas production technique (Pienaar, 1994) and *in vivo* data, to determine the predicted organic matter digestibility (OMD) of *A. nummularia* cv. De Kock, supplemented with different levels of maize and barley

| Levels | Treatment | |
|---|---|---|
| | **OMD %**<br>**Gas production technique** | **OMD %**<br>***In vivo* technique** |
| Atriplex 0% | $30.830_1$ [a] (±1.22) | $34.315_1$ [b] (±10.23) |
| Atriplex+15%Maize | $37.220_1$ [a](±0.10) | $54.676_2$ [b](±7.03) |
| Atriplex+15%Barley | $35.415_1$ [a](±0.12) | $61.283_2$ [b](±5.81) |
| Atriplex+30%Maize | $50.125_2$ [a](±2.44) | $55.692_2$ [b](±14.14) |
| Atriplex+30%Barley | $44.555_2$ [a](±0.70) | $62.448_2$ [b](±8.36) |
| Atriplex+45%Maize | $57.990_2$ [a](±0.69) | $63.954_2$ [b](±8.46) |
| Atriplex+45%Barley | $54.120_2$ [a](±0.48) | $67.033_2$ [b](±6.96) |

[ab] Row means with common superscripts do not differ (p>0.05)
[12] Column means with common subscripts do not differ (p>0.05)
(±) = standard deviation

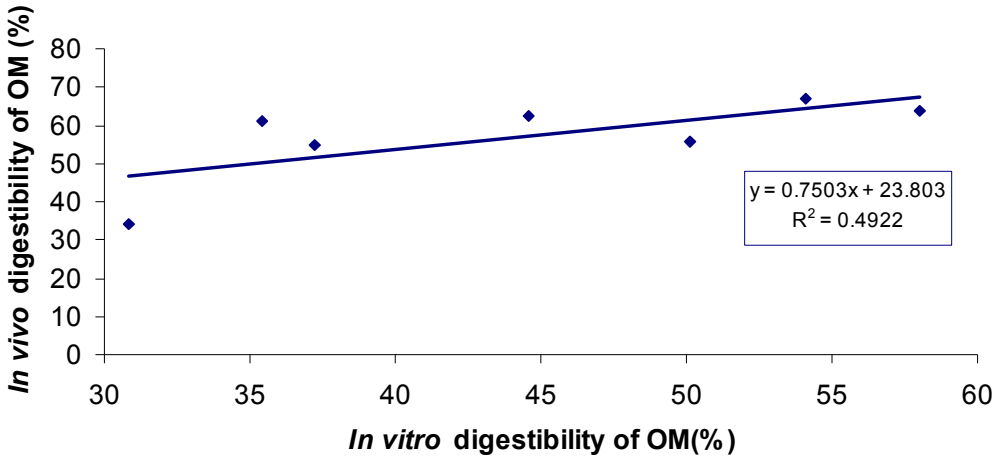


**Figure 6.3.1** The relationship between the *in vitro* gas production technique (Pienaar, 1994) and *in vivo* data, for the determination of a regression to predict organic matter digestibility (OMD) of *A. nummularia* cv. De Kock, supplemented with different levels of maize and barley

The $R^2$ of the above regression line is 0.492, which means that only 49% of the variance in *in vivo* digestibility, can be explained by the *in vitro* digestibility. A significant correlation (r=0.70) was, however, obtained between *in vivo* and *in vitro* digestibilities.

A linear regression equation, **y =0.7503x + 23.803**, could be fitted to the data.

Menke *et al*. (1979) first used the volume of fermentation products ($CO_2$ and methane) as a predictor of digestibility. Regression equations relate the volume of gas produced *in vitro* to the *in vivo* digestibility.

Khazaal *et al*. (1995) found $r^2$ values between *in vivo* digestibility and *in vitro* gas production ranged from 0.51 to 0.61. Menke and Steingass (1988), as cited by Nagadi *et al*. (2000b), also found a close correlation between gas production and *in vivo* digestibility, as did Mauricio *et al*. (2001).

Adesogan *et al*. (1998b) on the other hand, found that gas production gave a poor prediction of *in vivo* digestibility of a whole wheat crop ($r^2$ = 0.26). Menke

*et al*. (1979) suggested that inaccurate gas production and *in vivo* relationships could result from *in vitro* and *in vivo* differences, in the rates of degradation, outflow and improved post-ruminal utilisation of nutrient escaping fermentation. Other factors which may be implicated, include the indirect production of gas from the buffer or from microbial turnover, the dependence of gas production on the end products of fermentation and the microbial potential for switching between VFA and lactate production (Beuvink & Spoelstra, 1992).

An explanation of the correlation between gas production *in vitro* and digestibility *in vivo* can probably be found in the fact that this method is not based on a filtration process for separation of digested and undigested material. Such a separation may not give the full information on digestibility, partly because some indigestible or less digestible material may pass the filter. Some digestible material may not be extracted from the indigestible fraction on the filter (Menke *et al*., 1979).

In Figure 6.3.2 and Figure 6.3.3 a comparison is made between the different maize and barley levels of supplementation in the gas production *in vitro* technique (Pienaar, 1994) and the different levels of supplementation in the *in vivo* technique.

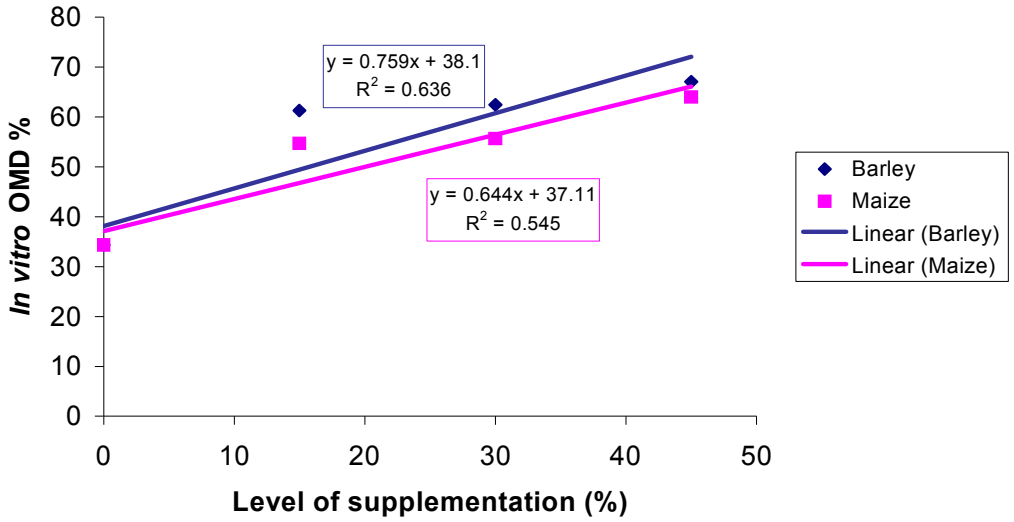


**Figure 6.3.2** A comparison between the different levels of maize and barley supplementation (%) of *A. nummularia* on the *in vitro* OMD %, with the *in vitro* gas production technique

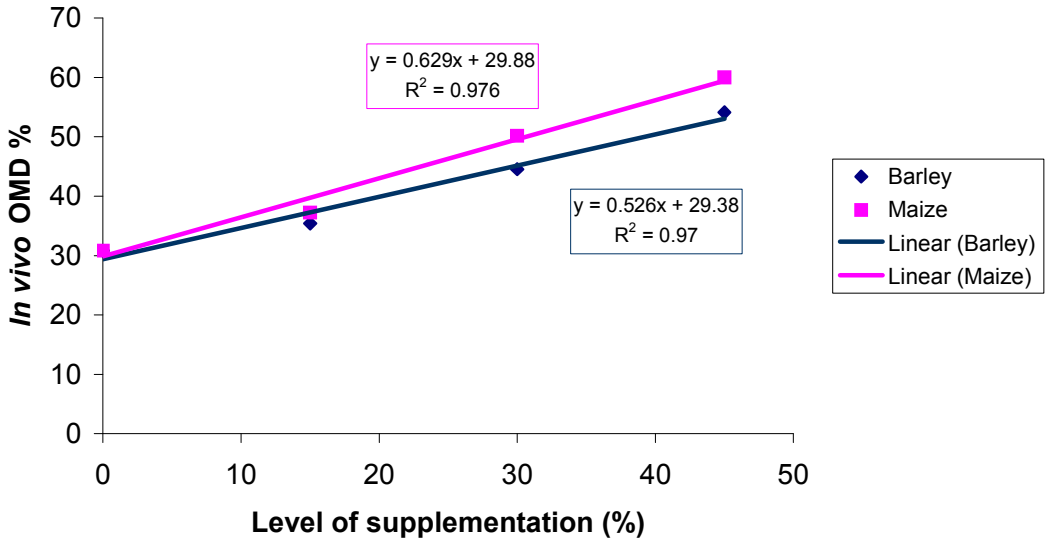


**Figure 6.3.3** A comparison between the different levels of maize and barley supplementation (%) of *A. nummularia* on the *in vivo* OMD %, with the *in vivo* technique

Table 6.3.2 shows the mean *in vitro* gas production OMD % compared to the mean *in vivo* OMD % over all levels of supplementation.

**Table 6.3.2** A comparison between the *in vitro* gas production technique and *in vivo* data, used to determine the organic matter digestibility (OMD) of *A. nummularia* cv. De Kock, over all the different levels of supplementation

| **Treatment** | **OMD %** |
|---|---|
| Gas production | 44.32[a] (±1.93) |
| *In vivo* digestibility | 56.05[b] (±1.93) |

[ab] Column means with common superscripts do not differ (p>0.05)
(±) = standard deviation

Gas production OMD % differs significantly from the *in vivo* OMD % values. There are several possibilities for the difference between the *in vivo* and *in vitro* OMD %. As with the previous two laboratory techniques, practical mistakes could have been made. Van Gelder *et al*. (2005) completed a ring test to determine laboratory repeatability and reproducibility of a gas production technique for measuring fermentation kinetics of feeds in rumen fluid. Repeatability refers to within laboratory variation and reproducibility between laboratory variations. These researchers found that within laboratory differences were influenced by variation in microbiological activity of the rumen fluid and air pressure changes at the end of the run. Varying microbial activity influences rate of fermentation and therefore, the time of incubation (Groot *et al*., 1996, as cited by Van Gelder *et al*., 2005). Microbial activity of rumen fluid inoculum from the same donor animal also varies with time. Part of this variation can be minimised by collecting rumen fluid from more than one donor animal and at fixed times relative to feeding. Higher microbial activity will result in shorter incubation time to reach a specific level of fermentation. Atmospheric air pressure is not constant. Pressure inside a closed bottle is measured relative to outside pressure, and with external pressure changes, inside pressure also changes. By including blank incubations, this effect can be compensated for, but not so much after 24h. Between laboratories, differences were observed in calibration factors, type of donor animal and diets fed. Calibrating factors and human operator effects go

hand in hand, and this will cause differences. Diet strongly affects rumen microbial flora (Nagadi *et al*., 2000b). In this study, the *in vivo* trial animals received the experimental diets, but the animals used to provide the rumen inocula for the gas production study received a lucerne and maize diet. It is preferable that samples are analysed in the rumen fluid, similar to the target *in vivo* situation.

The simulation of the rumen motility *in vitro* is often difficult and it may be that all the feed particles did not have the same exposure to the microorganisms, as it would have in the rumen of an animal. The different rumen pools are also not fully represented *in vitro*. Mould *et al*. (2005) stated that gastro-intestinal microflora exist in three sub-populations, which are those associated with the fluid phase, particulate material and gut epithelial tissue. Problems in sampling these niches may lead to poor estimation of microbial numbers and activity. Tafaj *et al*. (2005) found that the particle size of the feed also had an effect on the *in vitro and in vivo* microbial activity and fermentation patterns.

The fermentation characteristics and microbial constitution of the rumen inocula differ between the animals used for the *in vivo* digestibility trial and the animals used for rumen inocula collection. With *in vivo* digestibility the time of digestion is not known, and the time of rumen and gastric digestion *in vitro* could, therefore, have either been too long or too short.

Kitessa *et al*. (1999) concluded that the accuracy and precision of this method will depend on the magnitude of various variables. As far as inocula source is concerned, *in vivo* digestibility predicted from gas production should be susceptible to the same factors discussed with the rumen fluid *in vitro* technique. Gas leakage, effect of pressure from accumulated gas on gas solubility, size of incubation vessel in relation to space above substrate have also been mentioned as factors that may affect the fermentation kinetics of feeds (Cone *et al*., 1997).

**6.4 The *in vitro* cellulase technique (de Boever *et al.*, 1986) and the *in vitro* cellulase technique (Wageningen Institute of Animal Science, The Netherlands)**

To determine how accurate the *in vitro* cellulase digestibility techniques can be to predict, the organic matter digestibility (OMD) of *A. nummularia* cv. De Kock supplemented with different levels of maize and barley, a comparison between two cellulase techniques and the *in vivo* data is presented in Table 6.4.1 and Figures 6.4.1 and 6.4.2.

**Table 6.4.1** A comparison between two different cellulase techniques and *in vivo* data to determine the predicted organic matter digestibility (OMD) of *A. nummularia* cv. De Kock, supplemented with different levels of maize and barley

| Levels | Treatment | | |
|---|---|---|---|
| | **OMD % Cellulase - De Boever** | **OMD % Cellulase - Wageningen** | **OMD % *In vivo* technique** |
| Atriplex 0% | $34.000_1$ $^{ab}$(±0.29) | $36.896_1$ $^{a}$(±1.22) | $34.315_1$ $^{b}$ (±10.23) |
| Atriplex+15%Maize | $44.223_2$ $^{a}$(±1.60) | $48.846_2$ $^{a}$(±0.05) | $54.676_2$ $^{b}$(±7.03) |
| Atriplex+15%Barley | $46.739_2$ $^{a}$(±1.53) | $47.051_2$ $^{a}$(±0.50) | $61.283_2$ $^{b}$(±5.81) |
| Atriplex+30%Maize | $56.505_3$ $^{a}$(±1.03) | $48.734_2$ $^{a}$(±0.32) | $55.692_2$ $^{b}$(±14.14) |
| Atriplex+30%Barley | $53.117_3$ $^{a}$(±0.36) | $47.142_2$ $^{a}$(±0.40) | $62.448_2$ $^{b}$(±8.36) |
| Atriplex+45%Maize | $61.268_3$ $^{ab}$(±0.88) | $58.974_3$ $^{b}$(±1.19) | $63.954_2$ $^{b}$(±8.46) |
| Atriplex+45%Barley | $61.175_3$ $^{a}$(±0.74) | $58.729_3$ $^{a}$(±0.95) | $67.033_2$ $^{b}$(±6.96) |

$^{ab}$ Row means with common superscripts do not differ (p>0.05)
$^{123}$ Column means with common subscripts do not differ (p>0.05)
(±) = standard deviation

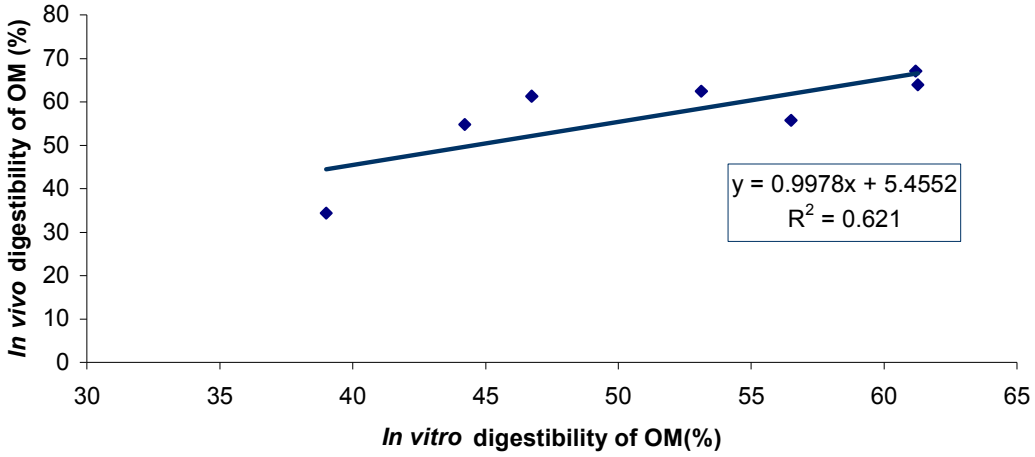


**Figure 6.4.1** Relationship between the *in vitro* cellulase technique (De Boever *et al*., 1986) and *in vivo* data, for the determination of a regression to predict organic matter digestibility (OMD) of *A. nummularia* cv. De Kock, supplemented with different levels of maize and barley

The $R^2$ of the above regression line is 0.621, which means that 62% of the variance in *in vivo* digestibility can be explained by the *in vitro* digestibility.
A significant correlation (r=0.79) was therefore, obtained between *in vivo* and *in vitro* digestibilities.

A linear regression equation, **y =0.9978x +5.4552**, could be fitted to the data.

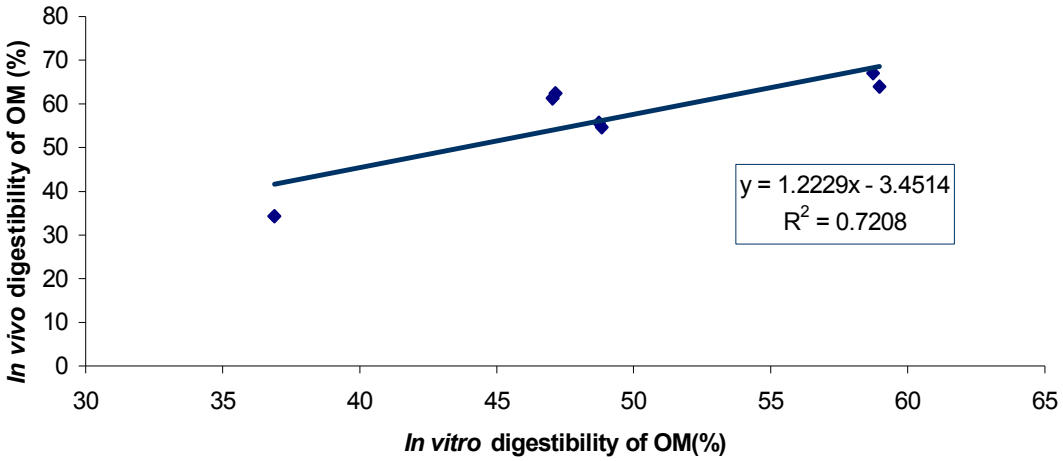


**Figure 6.4.2** A relationship between the *in vitro* cellulase technique (Wageningen) and *in vivo* data, for the determination of a regression to predict organic matter digestibility (OMD) of *A. nummularia* cv. De Kock, supplemented with different levels of maize and barley

The $R^2$ of the above regression line is 0.7208, which means that 72% of the variance in *in vivo* digestibility, can be explained by the *in vitro* digestibility. A highly significant correlation (r=0.85) was therefore, obtained between *in vivo* and *in vitro* digestibility's.

A linear regression equation, **y =1.2229x – 3.4514**, could be fitted to the data.

From the regression equation of the *in vitro* cellulose technique of De Boever *et al*. (1986) it can be seen that constant *a* has a value of -5.4552*,* which is small and *b* has a value of 0.9978 and is approaching 1. With the regression equation of the *in vitro* cellulose technique from Wageningen, constant *a* has a value of 1.2229 and *b* has a value of -3.4514, therefore, the assumption is made that the digestion was fairly complete.

Jarrige *et al*. (1970), as cited by Jones & Theodorou (2000), were some of the first to find solubility in the cellulase to be highly correlated (r=0.92) with *in vivo* digestibility for grass and legume forages. Several authors have adjusted the cellulase digestibility values to regression equations to obtain better

predictions of *in vivo* digestibility. Barber *et al*. (1984) found a $r^2$ value of 0.73 for fresh grasses. For by-products, Gasa *et al*. (1989) found a $r^2 = 0.78$.
Both Moss *et al*. (1990) and Givens *et al*. (1991) studied straws, and found $r^2$ values of between 0.44 and 0.51. De Boever *et al* (1986) found the $r^2$ value on concentrates to be 0.94.
Cellulose-pepsin solubility was highly correlated (r=0.87) with *in vivo* digestibility, but different regression equations were required for grasses and legumes (Jones & Theodorou, 2000). It was concluded that acid-pepsin treatment improved the accessibility of the cell-wall polysaccharides to the cell-wall-degrading enzymes.

In Figures 6.4.3, 6.4.4 and 6.4.5 a comparison is made between the different levels of maize and barley supplementation in the two *in vitro* cellulase techniques and the different levels of maize and barley supplementation in the *in vivo* technique.

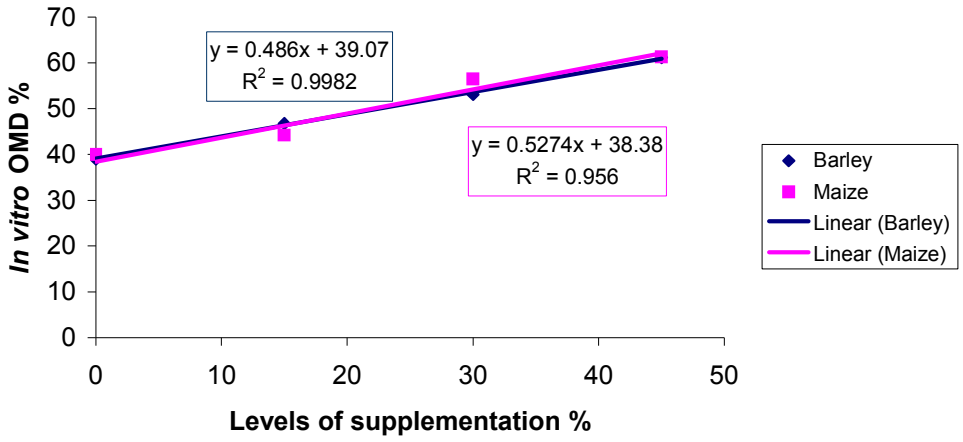


**Figure 6.4.3** A comparison between the different levels of maize and barley supplementation (%) of *A. nummularia* on the *in vitro* OMD %, with the *in vitro* cellulase technique (De Boever, 1986)

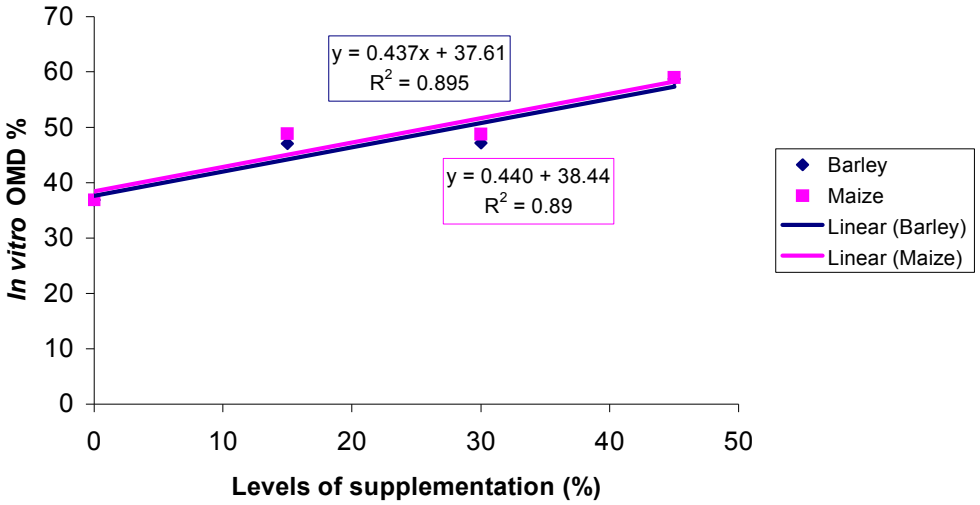


**Figure 6.4.4** A comparison between the different levels of maize and barley supplementation (%) of *A. nummularia* on the *in vitro* OMD %, with the *in vitro* cellulase technique (Wageningen)

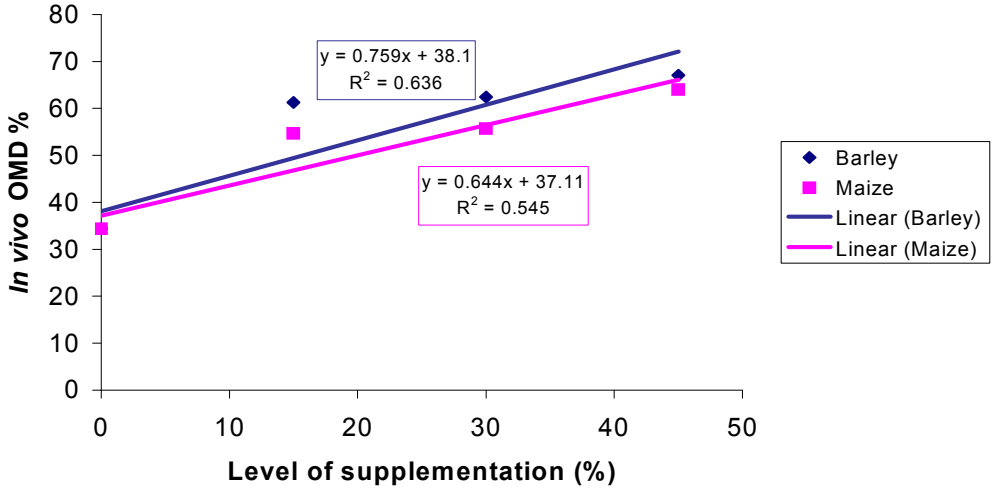


**Figure 6.4.5** A comparison between the different levels of maize and barley supplementation (%) of *A. nummularia* on the *in vivo* OMD%, with the *in vivo* technique

Table 6.4.2 shows the mean *in vitro* cellulase OMD % compared to the mean *in vivo* OMD % over all levels of supplementation.

**Table 6.4.2** A comparison between the two cellulase *in vitro* techniques and *in vivo* data, used to determine the organic matter digestibility (OMD) of *A. nummularia* cv. De Kock, over all the different levels supplementation

| Treatment | OMD % |
|---|---|
| Cellulase (De Boever) | $51.72^{ab}$ (±8.34) |
| Cellulase (Wageningen) | $49.48^{a}$ (±7.32) |
| *In vivo* digestibility | $56.05^{b}$ (±1.93) |

[ab] Column means with common superscripts do not differ (p>0.05)
(±) = standard deviation

Table 6.4.2 shows that the cellulase *in vitro* OMD % (Wageningen) differed significantly from the *in vivo* OMD % values, with cellulase *in vitro* OMD % (De Boever *et al*., 1986) being intermediate.

There are several possible explanations for the difference between the *in vivo* and *in vitro* OMD %. As with all the other techniques, practical mistakes could have been made. The simulation of the rumen environment *in vitro* is often difficult and with the use of enzyme preparations to determine digestibility, there isn't much scope for possible interaction between microbial species in the rumen and the modification of this by the diet of the host animal (Kitessa *et al*., 1999). This can also partly explain why the digestibility values are lower for low quality feeds (Morrison, 1983, as cited by Kitessa *et al*., 1999). The different rumen pools are also not fully represented *in vitro*. With *in vivo* digestibility the time of digestion is not known, and therefore, the time of rumen and gastric digestion *in vitro* could have been either too long or too short. This method also fails to account for the effects of level of feeding and particle size on *in vivo* digestibility (Kitessa *et al*., 1999). Stern *et al*. (1997) suggested that the biological validity of the results could be limiting as a result of incomplete enzymatic activity. The enzyme concentration must be sufficient to saturate the substrate, if enzyme concentration is limiting, accumulation of end products during incubation can lead to inhibition of enzyme activity.

# CHAPTER 7

## GENERAL CONCLUSIONS ON THE DIFFERENT TECHNIQUES USED TO DETERMINE THE DIGESTIBILITY OF *A. NUMMULARIA* CV. DE KOCK

The most accurate determination of nutritive value, as indicated by digestibility, dry matter intake (DM) and production, for animal feeds comes from *in vivo* feeding studies. However, high labour inputs, high cost and the need for large quantities of feed makes them unsuitable for routine feed evaluation. Prediction of digestibility of ruminant feeds using simple, reliable and inexpensive techniques is, therefore, important (Garcia-Rodriguez *et al*., 2005).

**Table 7.1** A comparison between the different *in vitro* techniques to determine which gives the best prediction of organic matter digestibility (OMD) of *A. nummularia* cv. De Kock supplemented with different levels of maize and barley

| Levels | Treatment | | | | |
|---|---|---|---|---|---|
| | OMD % Rumen fluid inoculum | OMD % Faeces fluid Inoculum | OMD % Gas production | OMD % Cellulase - De Boever | OMD % Cellulase - Wageningen |
| Atriplex 0% | 39.583[a](±1.202) | 38.233[a](±0.92) | 30.830[b] (±1.22) | 34.000[b](±0.29) | 36.896[b](±1.22) |
| Atriplex+15% Maize | 65.873[a](±0.143) | 65.152[a](±2.51) | 37.220[b](±0.100) | 44.223[b](±1.60) | 48.846[b](±0.05) |
| Atriplex+15% Barley | 62.444[a](±10.588) | 62.300[a](±1.50) | 35.415[b](±0.1.17) | 46.739[b](±1.53) | 47.051[b](±0.50) |
| Atriplex+30% Maize | 64.886[a](±2.063) | 63.159[a](±0.91) | 50.125[b](±2.44) | 56.505[b](±1.03) | 48.734[b](±0.32) |
| Atriplex+30% Barley | 69.554[a](±0.392) | 68.053[a](±6.43) | 44.555[b](±0.70) | 53.117[b](±0.36) | 47.142[b](±0.40) |
| Atriplex+45% Maize | 68.304[a](±0.100) | 66.253[a](±1.76) | 57.990[b](±0.69) | 61.268[b](±0.88) | 58.974[b](±1.19) |
| Atriplex+45% Barley | 72.49[a](±10.083) | 73.3495[a](±4.70) | 54.120[b](±0.48) | 61.175[b](±0.74) | 58.729[b](±0.95) |

[abc] Row means with common superscripts do not differ (p>0.05)
(±) = standard deviation

No significant differences (P>0.05) were found between the rumen- and faeces inoculum *in vitro* techniques, but differed significantly from the gas production and cellulase techniques. There was also no significant difference between the gas production and cellulase techniques. The OMD % of the *in vitro* techniques differed significantly from the *in vivo* OMD % values.

The results in Table 7.1 demonstrated that both the rumen- and faeces inoculum *in vitro* techniques could be used to determine the OMD of *A. nummularia* cv. De Kock, supplemented with an energy concentrate up to 45%. Higher *in vitro* OMD values will be the result, in comparison to the *in vivo* OMD values. This confirms that the *in vitro* faeces technique of El Shaer *et el*. (1987) is an easier and cheaper alternative to the classic rumen fluid *in vitro* technique of Tilley & Terry (1963), as modified by Engels & Van der Merwe (1967) to determine the OM digestibility of ruminant feeds.
The results also showed that the gas production and cellulase *in vitro* techniques resulted in lower OMD values than the *in vivo* technique. The two *in vitro* cellulase techniques are an alternative to the *in vitro* gas production technique of Pienaar (1994), to determine the OM digestibility of ruminant feeds. It eliminates the need for rumen inocula from cannulated animals. The cellulase *in vitro* technique, where starch is removed through hydrolysis (De Boever *et al*., 1986), has the advantage of being simpler and cheaper, because there is no need to add extra enzymes.

Adesogan (2002) recommended that with the current focus on measuring nutritionally related plant characteristics, future feed evaluation methods should determine the concentrations of specific nutrients in feeds and their rates of release to facilitate the prediction of animal response. In addition to refining current methods for the estimation of intake, microbial protein production and outflow rates, future analytical methods should aim to accurately determine diet selection, supplementation effects and associative effects of different ingredients fed together. Non-conventional factors such as the environmental impact of feeds and their potential for transferring bioactive compounds to humans should also be the focus of future evaluation methods (Givens, *et al*., 2000).

Kitessa *et al*. (1999) concluded that the *in vivo* digestibility of a feed is a function of the interaction between the feed and the animal, and the laboratory techniques predict

digestibility largely on feed sample information alone. Each of the techniques offer different features and choosing one method over another will, therefore, largely depend on the reason for measuring digestibility. Commercial laboratories and advisory services will benefit from choosing a method that has good repeatability and enables a large number of samples to be analysed per week. In research, the accuracy of prediction will determine the choice.

The technique first described by Tilley & Terry (1963), has been the most commonly used *in vitro* method for predicting digestibility and for use as a selection tool to improve the nutritional quality of forages (Stern *et al*., 1997). It is a convenient method to use, particularly when large-scale testing of feedstuffs is required (Getachew *et al.,* 1998). The Tilley & Terry (1963) technique is inexpensive and only small feed samples are required. The method shows promise to predict digestibility, even at levels of intake higher than the maintenance level. Digestibility of different parts of plants, such as leaves and stems and of cultivars in the early stages of development can be determined. A further possible application is in the evaluation of samples from oesophageal fistulated animals. From this study it was again confirmed that the *in vitro* Tilley & Terry (1963) method correlated well with *in vivo* digestibilities.

The main advantage of the faecal liquor technique is its simplicity combined with its minimal cost, when compared with the methods based upon rumen inocula. It uses freely available waste products from farm animals, managed under normal commercial conditions, saving the cost and inconvenience of maintaining fistulated ruminants.

Faecal liquor is useful when donor material must be obtained from free-ranging animals (Omed *et al*., 2000). Aiple *et al*. (1992) also found that although the hind gut of ruminants is regarded more as a fermentation chamber for structural carbohydrates escaping the rumen, inherent microbes are very adaptable and able to degrade non-structural carbohydrates too.
The materials needed are intact sheep for faeces, bottles, chemicals, carbon dioxide, a 39°C incubator, a centrifuge and a balance. The operator needs few chemistry skills. The faeces liquor procedure would, therefore, seem to be particularly suitable where potential food sources need to be screened, using limited equipment (Omed *et al.,* 2000).

This technique will probably be limited to centres that can't obtain rumen fluid, as it sometimes give lower digestibility values and combines the protracted, labour-intensive aspects of the rumen fluid-pepsin technique with faeces collection and activity problems (Adesogen, 2002).
Mauricio *et al*. (2001) have suggested that using faeces as inoculum, has the potential to estimate OMD *in vitro* as accurately as with rumen liquor and other end-point techniques. This was also concluded from the current study, with no significant differences between the *in vitro* OMD% of the rumen- and faeces inoculum techniques.

Kitessa *et al*. (1999) noted that this method is susceptible to the same sources of variation, as when using rumen liquor. In addition, obtaining the correct level of dilution with artificial saliva and standardising this concentration between runs will require more skill than with using rumen liquor. It was also suggested that the collection of faeces after feeding might be standardised by rectal grab sampling.

Enzymatic methods offered similar accuracy, but better repeatability, when compared to inocula-based methods. The pepsin-cellulase method is recommended for analysis of digestibility of feeds other than those with high starch content. For cereal grains or forage-cereal grain mixtures, the use of pepsin-cellulase plus amylase (or high temperature) or the Tilley & Terry (1963) method, is recommended. The pepsin-cellulase plus amylase is preferred for better repeatability, as it involves less biological variation than inocula based methods (Kitessa *et al*., 1999). Colombatto *et al*. (2000) examined the use of enzyme preparations to study the DM degradation dynamics of forages. It was concluded that the enzyme mixtures have the potential to describe the DMD profiles of maize silage and alfalfa hay, but that further research was needed for different feeds. Based on the review the, cellulase-based techniques reviewed, can predict *in vivo* digestibility more precisely than chemical methods, and are at least comparable in accuracy to inoculum digestion methods (Kitessa *et al*., 1999; Jones & Theodorou, 2000). In this study it was found that the two different cellulase techniques, De Boever *et al*. (1986) and Wageningen, did not result in significantly different OMD%. Both of the cellulose techniques had significant correlations with the *in vivo* OMD%, with the Wageningen cellulase technique correlation a little better that the De Boever *et al*. (1986) cellulase technique. This can be linked to the recommendation of

Kitessa *et al*. (1999) that for a forage-cereal grain mixture, as in the case of this study, *A. nummularia* with barley and maize, pepsin-cellulase plus amylase will result in a better prediction of *in vivo* OMD%.

For a method to have any real value in forage evaluation, it is essential that predictive equations are applicable to other populations, at least of the same or similar forages. The relationships between *in vivo* digestibility and enzyme solubility for different crops will be variable under different environmental and other conditions. It is known that environmental factors can influence both the content and degree of lignification of the cell wall. Enzyme methods solubilise less of the cell wall than *in vivo* digestion and are more influenced by the degree of lignification. It is likely, therefore, that environmental effects on cell-wall structure will influence enzyme solubility differently from *in vivo* digestion. Differences in botanical composition may also be significant, since it appears that the cell walls of legumes are more readily solubilised by cellulase than those of grasses (Jones & Theodorou, 2000).

A further consideration is that *in vivo* digestion can be influenced by factors such as level of feeding, particle size and length or the nutrient status of the feed (Kitessa *et al*., 1999; Jones & Theodorou, 2000). With silages, differential losses of volatiles or the degree and type of fermentation on rumen function and digestibility will also influence the predictive equations. There is clearly a need for further *in vivo* and analytical studies to determine the extent to which predictive equations derived from enzyme solubility apply to different environmental and other conditions (Jones & Theodorou, 2000).

Jones & Hayward (1973) found the solubility of dried grasses in *Trichoderma* cellulase to be highly correlated with *in vivo* digestibility. The amount of dry matter solubilised by the enzyme preparation used by Jones & Hayward (1973) was lower than the *in vivo* or *in vitro* (inoculum) values. A regression equation was, therefore, required to relate cellulase solubility to *in vivo* digestibility. Different regression equations were required for different materials assayed, but, subject to this provision, cellulase solubility was considered a suitable alternative to inoculum methods (Jones & Theodorou, 2000).

Of the cellulase methods currently in use, the pepsin-cellulase method has been found to be the least time consuming and easiest to manipulate (Downman & Collins, 1982). It also uses inexpensive glassware and chemicals and a higher output per person can be achieved, than with methods involving refluxing with detergent or strong acid.

Generally, predictions based on the *in situ* technique were more accurate an explained a greater percentage of variation in the *in vivo* DMD, than the *in vitro* rumen liquor-pepsin procedure. Both in theory and practice, this method is the closest to measurement of *in vivo* digestibility. The accuracy of the other methods could be tested or adjusted against the *in situ* method, if *in vivo* trials are not practical (Kitessa *et al*., 1999).

The *in vitro* gas production procedure in the current study did not exceed either the enzymatic or inocula-based procedures in accuracy of predicting total tract DM or OM digestibility. However, it can be used in screening cereal grains for the rate of hydrolysis of starch in the rumen, which is crucial in the digestion of forage-grain mixtures by ruminants. For this purpose it is recommended in preference to the *in situ* method, which is more laborious and more variable (Kitessa *et al*., 1999). Mould *et al*. (2005) mentioned that the extent of rumen degradation of a feedstuff is the product of ruminal residence time and rate of degradation. The Tilley & Terry (1963) two-stage technique is deficient in that it does not provide kinetic information. It is, therefore, possible for two feeds to have similar end-point degradation values, but different rates of degradation. This is of fundamental importance, as feeds with higher degradation rates tend to be consumed in greater quantity. In addition, these assays are generally made after an incubation period of 48-96h. It is, therefore, possible that degradation is over-estimated and subtle differences between similar feeds lost. The main advantage of this method, compared to the rumen-liquor and enzyme methods, is that it takes less time and totally removes errors due to washing, filtration and weighing of residues. The method is potentially susceptible to underestimating digestibility of feeds that are slow in producing gas, but which have a high *in vivo* digestibility (Kitessa *et al*., 1999). Adesogan (2002) also stated that results of gas production experiments are often misinterpreted and used to draw inappropriate deductions. Rymer *et al*. (2005) suggested that if the gas production technique is to be used as a means of feed evaluation, it may be necessary to require a standardised particle size and sample

preparation procedure in order to reduce variation between experiments and laboratories.

To conclude, from the current study, the accuracy of all the *in vitro* methods was lowest when predicting the *in vivo* digestibility of low quality feeds, such as hay and cereal by-products. It is likely that the effect of passage rate and level of intake on digestibility, as well as, animal variation, is greatest on such feed types. None of the *in vitro* methods can fully account for these variables (Kitessa *et al*., 1999).

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### 6.5 Dry matter digestibility

The digestibility of pure *A.nummularia* (0% supplementation) as reported in this trial was lower than those reported by other researchers (Hassan & Abd El-Aziz, 1979). Atiq-ur-Rehman *et al*. (1995) reported that digestibilities could range from 47% to 74% when fed to sheep. The lower digestibilities found in the present trial, may be due to the higher stem to leaf ratio and differences in the amount lignin found in the stems. The material used in this trial was collected from older plants, which had been grazed in the winter prior to harvest. Therefore, the residual material tended to be more fibrous and less palatable.

The increase in dry matter digestibility as energy supplementation is provided supports the results reported by previous authors (Ørskov, 1982, Warren & Casson, 1993). The increase in digestibility, with energy supplementation, can be explained by the higher content of digestible organic matter in the energy sources. The differences in digestibility at the different levels could, in addition, be due to associative effects.

Mould *et al*. (2001) studied the non-additivity of feedstuffs *in vitro* and the influence of incubation medium pH. Non-additivity occurs when the nutritive value of mixed feedstuffs differs from that of the sum of its components. One dietary constituent can have an influence, positive or negative, on the apparent digestibility of another, where other components, such a nitrogen and sulphur are non-limiting. Negative effects occur due to the depression of rumen pH or substrate competition and positive effects occur when a readily fermentable fibre source has been combined with poorly fermented forages. The authors observed that non-additive effect can be identified with the use of an *in vitro* gas production technique and that the magnitude varies with incubation interval and pH of the fermentation medium. The results suggest that the use of gas production and OMD from single feeds, to generate estimations for a combined feed would not be valid. Sandoval-Castro *et al*. (2000) also studied *in vitro* associative effects of feed mixtures, with the *in vitro* gas production technique. It was found the IVDMD and gas production was depressed when feed mixtures were tested.

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## APPENDIX

The gas production system at the Agricultural Research Centre – Animal Improvement Institute, Irene.

The pressure sensors at the Agricultural Research Centre – Animal Improvement Institute, Irene.

The centrifuge at the Agricultural Research Centre – Animal Improvement Institute, Irene.

Collection of rumen fluid of sheep at the Agricultural Research Centre – Animal Improvement Institute, Irene.

Rumen fluid and artificial saliva mixture flushed with carbon dioxide. It is then added to the samples in the Scott reagent bottles, and then closed with the pressure sensor caps