Development of the in vitro gas production technique to estimate protein degradation in the rumen

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DECLARATION

I hereby declare that the work presented in this thesis is the product of my own efforts and has not been submitted in any previous application for a degree or professional qualification. The work on which it is based is my own except where stated in the text and in the acknowledgements section.

30th August, 2006

Matthew Palmer
ABSTRACT

The purpose of this study was to adapt the in vitro gas production technique (IVGPT) to estimate the rumen degradation profile of feed protein and to investigate the effect of sample preparation on the estimated kinetics of silage carbohydrate and protein degradation in the rumen.

The effects of drying during sample preparation and the sample particle size were shown to have significant effects on the carbohydrate degradation characteristics of fresh silages. Indirect gas produced by silage fermentation acids (SFA) occurs in the first 30 minutes of incubation and although reduced by drying silages still causes large bias in the overestimation of quickly fermentable carbohydrate (QCHO) and totally fermentable carbohydrate (TCHO). Fresh silages contained significantly more TCHO than dried material with a higher concentration of QCHO in fresh grass silage and a higher concentration of slowly fermentable carbohydrate (SCHO) in maize silage and wholecrop wheat. Although there was no effect on lag (P>0.05), SCHO fermented significantly slower in fresh grass silage (P<0.01) and fresh maize silage (P<0.001) compared to the dried material. Only 80.3% of soluble silage carbohydrate is quickly fermented and 19.7% of QCHO is from insoluble carbohydrates sources.

IVGPT was adapted to measure protein degradation by incubating a weight of sample equivalent to 78.4mgN/l with 100mg of sucrose in a nitrogen free IVGPT environment. The availability of nitrogen was shown to have a significant positive effect on the gas produced during IVGPT in a rumen environment up to 78.4 mgN/l but at higher ammonia concentrations there was a drop in gas production (P<0.05). Zeolite was used to remove ammonia from rumen liquor with a minimum absorbance coefficient of 1mgNH₄-N/g and with no detrimental effect on microbial activity (P>0.05).

The IVGPT estimated concentration of quickly degradable protein (QDP) in grass silage was significantly lower than that estimated using the nylon bag technique (P<0.001), which also overestimated the concentration of soluble nitrogen by 23%. The IVGPT estimates of QDP were positively correlated to soluble nitrogen availability (R²=77.9, P<0.001) and indicated that only 76.3% of soluble nitrogen is quickly degraded.
During oven drying there was no loss of soluble free amino acids (P>0.05) from fresh material but using dried and milled material significantly underestimated the protein A fraction (P<0.05) and QDP concentration (P<0.05) whilst overestimating the protein B fraction (P<0.05) in grass silage and maize silage.
Acknowledgements

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I am also forever indebted to all those who helped me in the laboratory and for their patience when my ideas didn’t work. To Lynsey Doyle for her assistance during the numerous gas runs, to Iris Chanay for being a star and to Adrian Gonzalez and Astrid Marquez muchas gracias por tus ayuda y amistad.

Finally, a special thanks to my ‘confident’ Mr Graeme Allan, the man with all the answers and whom without none of this would have ever left the ground. Gas run next week Graeme?!
‘All things being equal, the simplest answer is usually the right one’

WILLIAM OCKHAM

‘Anything is possible’

OM MANI PADME HUM - MANTRA

To Aoife
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<tr>
<td>ABM</td>
<td>Anaerobic Buffer Medium</td>
</tr>
<tr>
<td>ADF</td>
<td>Acid Detergent Fibre</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Tri Phosphate</td>
</tr>
<tr>
<td>Ca</td>
<td>Rate of fermentation of QCHO or QDP (h⁻¹)</td>
</tr>
<tr>
<td>Cb</td>
<td>Rate of fermentation of SCHO or SDP (h⁻¹)</td>
</tr>
<tr>
<td>CNCPS</td>
<td>Cornell Net Carbohydrate and Protein System</td>
</tr>
<tr>
<td>COD</td>
<td>Chemical Oxygen Demand</td>
</tr>
<tr>
<td>CP</td>
<td>Crude Protein</td>
</tr>
<tr>
<td>DM</td>
<td>Dry Matter</td>
</tr>
<tr>
<td>DMD</td>
<td>Dry Matter Digestibility</td>
</tr>
<tr>
<td>DMI</td>
<td>Dry Matter Intake</td>
</tr>
<tr>
<td>DUP</td>
<td>Digestible Undegraded Protein</td>
</tr>
<tr>
<td>ERDP</td>
<td>Effective Rumen Degradable Protein</td>
</tr>
<tr>
<td>FME</td>
<td>Fermentable Metabolisable Energy</td>
</tr>
<tr>
<td>GLM</td>
<td>General Linear Model</td>
</tr>
<tr>
<td>IVGPT</td>
<td>In Vitro Gas Production Technique</td>
</tr>
<tr>
<td>ME</td>
<td>Metabolisable Energy</td>
</tr>
<tr>
<td>MP</td>
<td>Metabolisable Protein</td>
</tr>
<tr>
<td>MPS</td>
<td>Microbial Protein Synthesis</td>
</tr>
<tr>
<td>NDF</td>
<td>Neutral Detergent Fibre</td>
</tr>
<tr>
<td>NDIN</td>
<td>Neutral Detergent Indigestible Nitrogen</td>
</tr>
<tr>
<td>NFAL</td>
<td>Non Fermentative Acid Loading</td>
</tr>
<tr>
<td>NIRS</td>
<td>Near Infrared Reflectance Spectroscopy</td>
</tr>
<tr>
<td>NPN</td>
<td>Non Protein Nitrogen</td>
</tr>
<tr>
<td>OM</td>
<td>Organic Matter</td>
</tr>
<tr>
<td>QCHO</td>
<td>Quickly Degradable Carbohydrate</td>
</tr>
<tr>
<td>QDP</td>
<td>Quickly Degradable Protein</td>
</tr>
<tr>
<td>SCHO</td>
<td>Slowly Degradable Carbohydrate</td>
</tr>
<tr>
<td>SDP</td>
<td>Slowly Degradable Protein</td>
</tr>
<tr>
<td>SDNAN</td>
<td>Slowly Degradable Non Ammonia Nitrogen</td>
</tr>
<tr>
<td>SFA</td>
<td>Silage Fermentation Acid</td>
</tr>
<tr>
<td>SNAN</td>
<td>Soluble Non Ammonia Nitrogen</td>
</tr>
<tr>
<td>SNPN</td>
<td>Soluble Non Protein Nitrogen</td>
</tr>
<tr>
<td>TCHO</td>
<td>Totally Degradable Carbohydrate</td>
</tr>
<tr>
<td>TMR</td>
<td>Total Mixed Ration</td>
</tr>
<tr>
<td>VFA</td>
<td>Volatile Fatty Acid</td>
</tr>
</tbody>
</table>
1.1 INTRODUCTION

Feed represents the largest single cost in all ruminant production systems (De Boer and Bickle, 1988) and the balance between feed cost and nutrient supply is a crucial component in determining farm profitability. Nutritional management is particularly relevant in intensive high yielding dairy herds where rations need to be formulated to maximise milk production and nutrient supply whilst also optimising rumen fermentation and function. This requires knowledge of rumen physiology and herd management but most importantly an accurate description of dietary components. The appropriate assessment of feeds and their rate of fermentation and/or digestion in the ruminant is key to successful diet formulation both in terms of nutrient supply and diet cost.

Historically the kinetics of feed degradation in the rumen have been estimated using two main techniques. The first involves incubating feed in nylon bags placed in rumen liquor and measuring the disappearance of nitrogen or dry matter (DM) over time Ørskov and McDonald (1979). The second involves incubating feed in rumen liquor and measuring the cumulative gas produced over time (Menke and Steingass, 1988). This second technique is known as the in vitro gas production technique (IVGPT) and has been most successfully in measuring the kinetics of carbohydrate degradation in the rumen because it most notably provides a measure of the rate of fermentation of quickly degradable feed components. This is an advantage over the nylon bag technique which provides no such kinetic data and assumes that all water soluble feed components are immediately degraded, even though this fraction may also contain insoluble fine feed components washed from the nylon bag. The success of IVGPT in providing a measure of the kinetics of carbohydrate degradation in the rumen is however undermined by the lack of similar parameter estimates for feed protein which continues to be estimated using the nylon bag technique.

An additional source of contention is that in an attempt to produce a homogenous sample, feed samples are commonly heat dried and then milled to 1-4mm prior to incubation with rumen microbes. The pre-treatment of feed in this manner not only alters the physical characteristics of the fresh material (Poppi et al., 1981) but also alters its chemical composition (Pichard
and Van Soest, 1977; Acosta and Kothmann, 1978; Van Soest, 1982; Piccaglia and Galleti, 1987; Abdalla et al., 1988; Chamley and Viera, 1990; Snyman and Joubert 1992; Denium and Maassen, 1994; Ljókel et al., 2003). Consequently, analysis of the rate and extent of degradation of these feeds estimated after they have been dried and milled may provide misleading results (Nagadi et al., 1998; Lowman et al., 2002).

This thesis is concerned with the adaptation of IVGPT to estimate the ruminal protein degradation kinetics of UK silages and to study the effect of sample processing on the rumen degradation kinetics of silage carbohydrate and protein.

Each chapter of this thesis is written as a separate unit with each proceeded by an introduction outlining background information relevant to the subject area investigated. The following literature review is therefore only a general outline of the entire thesis.

1.2 LITERATURE REVIEW

1.2.1 THE RUMINANT

The success of the ruminant in agricultural production systems is due primarily to its ability to convert indigestible plant materials into highly digestible and tradable commodities such as meat and milk.

Up to 60-80% of plant organic matter (OM) is comprised of structural carbohydrates such as cellulose, hemicellulose and lignin (Chenost and Kayouli, 1997). The most abundant of these structural carbohydrates is cellulose which accounts for 32-47% of forage dry matter (DM) (Chenost and Kayouli, 1997) but is effectively indigestible in the mammalian gut due to a lack of enzymes capable of hydrolysing the β-bonds between the glucose units of cellulose (Hoover and Miller-Webster, 1997). In the ruminant, feeds are firstly fermented by microbes in the reticulo-rumen before entering the abomasum (true stomach). These rumen microbes secrete a range of enzymes to aid in the breakdown of feed particles including esterases, lipases, proteases, ureases (Moharrery and Das, 2001) and most importantly the hydrolytic enzymes capable of breaking down and digesting mono and polysaccharide carbohydrate complexes (Chenost and Kayouli, 1997). Such hydrolytic enzymes include glucosidase, mannosidase, xylosidase, amylase and hemicellulase (Williams and Strachan, 1984) but the most important is
cellulase which accounts for 60-90% of all carbohydrate fermented in the rumen (Chenost and Kayouli, 1997).

Although the ruminant does not benefit directly from the carbohydrates fermented by these rumen microbes, the volatile fatty acids (VFA) released as an end-product are absorbed across the rumen wall and can provide up to 80% of a ruminants energy requirements (Ishler, Heinrichs and Varga, 1997). As well as providing a means of accessing the chemical energy stored in plant material, rumen microbes are also a vital source of protein. With a true protein content of 75% and a digestibility of 85% (Chenost and Kayouli, 1997) microbes can provide up to three quarters of the total amino acids absorbed by the ruminant (Dewhurst, Davies and Merry, 2000).

1.2.2 HISTORY OF FEED ANALYSIS

Ever since animals were first domesticated in 40,000 to 10,000 B.C (Flannery, 1965) feeds have historically been evaluated, marketed and fed on the basis of their appearance and weight, a technique which, although seemingly effective, is also extremely subjective. The first standardised feed evaluation system was developed in Germany in the early 1800’s. This system originally assessed the feeding value of feeds against that of straw, which was the most important winter feed at that time, but by the late 1800’s the system had been revised with the standard straw unit replaced with hay (Morton, 1855). Unfortunately hay proved to be too variable and in the 1900’s the Kellner starch equivalent system (Kellner and Kohler, 1900) was adopted. This system proved very popular and up until very recently was the principal system used in Europe where it was adapted with feeds assessed against the feeding value of grains, most notable, barley and oats (Ørskov, 1998).

The nutritional value of ruminant feeds is highly dependent on its digestibility in the rumen, a factor related to the rate of microbial degradation and passage rate out of the rumen (Ørskov, 1998). As the genetic potential of the UK dairy herd increased following the widespread use of North American Holstein semen in the 1970’s, the level of attainable milk production began to exceed the potential of the rumen to support it. In order to maintain rumen function whilst also maximising dry matter intake (DMI) and milk production, an important component of ration formulation became the incorporation of feeds that resist microbial fermentation and which are digested directly in the abomasum. Although the equivalence systems were useful in standardising feeding values, they provided no guide
as to the rumen degradation characteristics of feeds and therefore limited the nutritionists ability to quantitatively balance diets to optimise rumen function and nutrient availability (Ørskov, 1998).

The proportion of feed able to escape rumen degradation to be absorbed in the small intestine is referred to as the ‘by-pass’ fraction and is highly variable both between and within plant species, especially in forages (Dale, 1996; Hoffman and Brehm, 1999). By-pass feed components enable diets to be formulated with levels of carbohydrate and protein that if fermented solely in the reticulo-rumen could induce acidosis and/or ketosis and have a detrimental effect on animal performance.

The variation in by-pass components of forages is further compounded when they are conserved for winter or buffer feeding. This is most evident in the degradation characteristics of forage proteins which during ensiling are broken down by microbial and forage proteases (Zhu et al., 1999) into smaller soluble and more rapidly degradable fractions composed principally of free amino acids and ammonia (Givens and Rulquin, 2004). Although the extent of proteolytic breakdown is dependent on management factors such ground condition, crop maturity, cutting date, dry matter, wilting time, degree of processing and compaction in the silo (Chamley and Veira, 1990; Hoffman and Brehm, 1999) it has been estimated that only 9-15% of macro forage protein (>10kDa) remains after fermentation (Messman et al., 1994). As a result, ammonia accounts for 12.1% of the total nitrogen in grass silage, 14.4% in red clover silage and 7.1% in maize silage (Givens and Rulquin, 2003). Ensiled forages therefore contain significantly less by-pass protein than non-ensiled forages and pose a considerable challenge when formulating Total Mixed Rations (TMR) or buffer rations for high yielding dairy cows. Conservation of wholecrop forages and grain for winter or buffer feeding can also effect the degradation characteristics of carbohydrates, most notably starch. Again, the solubility and rate of degradation of starch is fundamentally dependent on the plant species, maturity and cultivar but it is the method (Table 1.1) and degree of sample processing that ultimately determines microbial access and the rate of starch degradation in the rumen.
Table 1.1. Comparison of the effect of processing method on the microbial degradation of starch in the rumen. Adapted from Hoover and Miller-Webster (1997).

<table>
<thead>
<tr>
<th>Plant Species</th>
<th>Dry rolled</th>
<th>Steam rolled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>75</td>
<td>85</td>
</tr>
<tr>
<td>Barley</td>
<td>79</td>
<td>85</td>
</tr>
<tr>
<td>Wheat</td>
<td>88</td>
<td>88</td>
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<td>Oats</td>
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</table>

The efficiency of microbial utilisation of dietary protein for microbial protein synthesis (MPS) depends on the availability of energy in the rumen and the proportion used for microbial maintenance. At slow rates of microbial growth a greater proportion of dietary energy is used to meet maintenance requirements, whilst at faster growth rates a greater proportion of energy is used for growth, resulting in greater MPS efficiency and a higher yield of microbial protein (Dewhurst et al., 2000). In addition to carbohydrate availability, the efficiency of dietary nitrogen capture and MPS in rumen microbes also depends on the quantity and type of dietary nitrogen in the ration. Low efficiencies of dietary nitrogen capture have been associated with high nitrogen intake (Givens and Rulquin, 2003) whilst conversion of feed nitrogen to milk nitrogen has also shown to vary from 18% in lucerne silage (Dewhurst et al., 2006) to 23-32% in grass silage (Dewhurst et al., 1996).

Rumen microbes derive the ammonia, amino acids and peptides required for MPS from microbial degradation and proteolysis of dietary protein, non-protein nitrogen (NPN) and microbial protein or hydrolysis of recycled urea (Ishler, Heinrichs and Varga, 1997). Although ammonia nitrogen is the main source of nitrogen for most rumen microbes including cellulolytic and amylolytic bacteria (Chenost and Kayouli, 1997), the efficiency of MPS is enhanced by the addition of certain amino acids and peptides (Griswold, Hoover, Miller, Thayne, 1996).

These effects are further enhanced by adding readily fermentable carbohydrates such as soluble sugars (Chamberlain et al., 1993) or starch (Guzzon et al., 1999; Clammell et al., 2000) to diets. Miller et al., (2001) showed that cows grazing high sugar perennial ryegrass used nitrogen more efficiently and excreted less in the urine, compared to cows grazing a standard perennial ryegrass variety. These authors also showed that those cows grazing the high sugar grass also had higher digestible DM intakes and
higher milk yields, further demonstrating the importance of synchronising carbohydrate and protein availability in the rumen to maximise MPS and diet utilisation.

1.2.3 THE UK FEEDING SYSTEM

First proposed in 1965 to overcome the limitations of equivalent systems, the UK Metabolisable Protein (MP) system (AFRC, 1993) was designed to improve the accuracy of diet formulation by recognising the importance of carbohydrate and protein availability in the rumen in determining microbial protein synthesis (MPS) and nutrient supply.

The proportion of dietary Metabolisable Energy (ME) available in the rumen for MPS is referred to in the MP system as Fermentable Metabolisable Energy (FME) and is determined by subtracting the caloric content of VFA’s and fats, which it is assumed are not utilised by rumen microbes (AFRC, 1993, Deaville and Givens, 1998). Unfortunately, the MP system provides no measure of the kinetics of carbohydrate or FME availability in the rumen and there is therefore no quantitative measure of the risk of fermentative acidosis or the proportion of by-pass carbohydrate present in a ration. In contrast, the MP system does provide a measure of the kinetics of protein degradation in the rumen. This is normally estimated by placing feed in nylon bags and incubating them in the rumen of fistulated animals or in buffered rumen fluid and measuring the disappearance of nitrogen over time (Ørskov et al., 1980). The degraded protein is then categorised into two fractions; soluble or quickly degradable protein (QDP) and slowly degradable protein (SDP). This approach assumes that all nitrogen lost from the bag is soluble and immediately degradable but due to the relatively large pore size of the bags used (1600μm) soluble feed components, oil, starch and fine particles can also be washed out of the bags undegraded and thus contribute to the overestimation of protein degradation (Chen et al., 1987). This is further supported by other authors who have shown that this soluble protein fraction is not immediately degraded in the rumen (Fahmy et al., 1991; Aufrère et al., 1994) and up to 10-11% may escape degradation and contribute to the by-pass protein fraction (Aufrère et al., 2001; Choi et al., 2002; Volden et al., 2002).

Additional limitations of the nylon bag technique include microbial colonization of feed particles during incubation and failure to differentiate between undegraded feed protein and microbial protein (Broderick, 1987). Microbial colonisation occurs in the first 8 hours of incubation and after 48
hours microbial protein can account for up to 72% of the residual protein remaining in the bag (Alexandrov, 1998). To avoid the underestimation of protein degradation (Negi et al., 1988; Becker et al., 1995), laboratories commonly employ a post-incubation wash in an attempt to remove this residual microbial protein, but there is concern that these washing procedures may also remove feed protein (Tuori et al., 1998). Alternative methods developed to correct for microbial protein include using internal and external microbial markers (Broderick and Merchen, 1992) and incubating nitrogen free blanks (Negi et al., 1988), but these are expensive and tedious with a poor level of repeatability.

In an attempt to reduce the dependence on using rumen microbes, the Cornell Net Carbohydrate and Protein System (CNCPS) was developed to categorise carbohydrate and protein into chemically distinct fractions each with a specific fermentation rate (Sniffen et al., 1992). Although chemical assays are more accurate, the number of assays required to characterise each fraction makes routine analysis arduous and expensive (Hoffman et al., 1999).

1.2.4 IN VITRO GAS PRODUCTION TECHNIQUE (IVGPT)

Microbial degradation of carbohydrates in the rumen results in the production of volatile fatty acids (acetate, propionate and butyrate), carbon dioxide and methane (Hungate, 1966). This means that the rate of gas production can be used as an index of carbohydrate degradation.

1.2.4.1 THE HISTORY OF IVGPT

The principle of measuring gas production to assess microbial activity was first introduced by McBee (1953) and the original manometric technique continued to be developed over the years to assess the rumen degradation characteristics of feeds (Hungate et al., 1955; Czerkawski and Brekenridge 1970; Wilkins 1974). However, it was not until 1979 that a technique was developed involving incubating feeds in air tight syringes and measuring the cumulative gas produced over time, that the kinetics of carbohydrate degradation could be routinely estimated (Menke et al., 1979).

Rather than estimating feed degradation as the disappearance of organic matter (OM) from nylon bags, this technique which became known as the in vitro gas production technique (IVGPT) (Menke and Steingass, 1988) measures the end products of fermentation. It therefore has the potential to provide a better description of microbial degradation in the rumen, most
notably due to its ability to estimate the quantity and rate of fermentation of quickly degradable carbohydrate (QCHO). IVGPT has been successfully used to study the effect of intrinsic anti-nutritional factors in feeds such as tannins and saponins (Makkar et al., 1995), to predict ME (Menke and Steingass, 1988; Krishnamoorthy et al. 1995), dry matter digestibility (DMD) (Blümmel and Ørskov 1993) and neutral detergent fibre (NDF) digestibility (Herrero and Jessop, 1996).

In addition to providing a direct measure of ruminal carbohydrate degradation, the potential of IVGPT to be operated without the use of fistulated animals means that it also avoids the associated cost and welfare implications of using live animals. An additional commercial advantage of IVGPT is that because it involves the continual incubation of one feed sample, the degree of experimental error is significantly less than that in nylon bag technique which involves the incubation of numerous sub-samples in a number of animals. Similarly whilst the nylon bag technique is limited by the number of available animals, IVGPT is only limited by the number of available syringes and can therefore analyse a greater number of samples in any given space of time.

1.2.4.2 LIMITATIONS OF IVGPT

In principle the only gas measured during IVGPT is as a result of microbial degradation of feed carbohydrate, either directly as carbon dioxide and methane or indirectly as carbon dioxide released when the VFA’s produced during fermentation (Acetic, butyric and propionic acid) react with the bicarbonate buffer. Unfortunately, gas is also produced from a number of other sources meaning that microbial fermentation of carbohydrate accounts for only a proportion of the total gas observed.

1.2.4.2.1 ENDOGENOUS CARBOHYDRATE AND MICROBIAL TURNOVER

Up to 30% of the total gas observed during incubation can be attributed to microbial fermentation of feed particles already present in the rumen fluid and from turnover of microbial biomass (Cone et al., 1997). To account for this contribution of gas it is common to incubate blank syringes containing inoculum with no sample and to then subtract, at each time point, the gas produced by these blanks from that produced by each sample. Microbial turnover, which can account for 30% of the gas produced by the blanks, usually occurs around 1 hour after incubation when no sample is incubated but can occur much later in the presence of substrate (Cone et al., 1997). This
means that the rate of gas production by the blanks may not accurately reflect the rate of microbial turnover in the presence of the feed sample. To account for the unknown rate of microbial turnover in the presence of the feed it has been suggested that the microbial lag is either assumed to be 24 hours (Jessop, 2004) or no correction made for the blank at all (Cone et al., 1997).

An alternative approach is to reduce the contribution of the blank to total gas production by using a more dilute inoculum (Pell and Schofield, 1993). Unfortunately, the gas produced in high dilutions of ruminal fluid is not only dependent on the quantity of incubated substrate but also on the microbial population density with a greater proportion of the carbohydrate used for maintenance and growth rather than fermentation to VFA (Jessop and Herrero, 1998; Nagadi et al., 2000). The result is that feeds incubated in high dilutions of rumen fluid have a significantly lower rate and level of gas production and have longer lags compared to those incubated in lower dilutions (Nagadi et al., 2000).

1.2.4.2.2 OTHER FEED COMPONENTS

In addition to carbohydrates, other components of the incubated feed sample can also contribute to the total volume of gas produced by microbial fermentation.

The contribution of protein fermentation to total gas production is usually ignored as it is assumed to be small (Wolin, 1966) and primarily restricted to the first hours of incubation (Cone et al., 1997). The effect of oil on the total gas production is also assumed to be negligible (Menke and Steingass 1988) but because high concentrations are toxic to rumen microbes it has been recommended that oil is extracted from high oil feeds (>100g/ kgDM) before incubation (Moss et al., 1998).

Because anaerobic fermentation of plant organic matter during ensiling also produces VFA and lactic acid, it has been suggested by Deaville and Givens (1998) that silage fermentation acids (SFA) may affect the gas production profile of feeds by indirectly reacting with the bicarbonate buffer. As a result Deaville and Givens (1998) suggested that all samples should be pre-dried in an oven prior to incubation and although duly adopted the impact of drying on the interference of silage SFA has not been demonstrated.
1.2.4.2.3 VFA STOICHIOMETRY

Although the gas production profile during IVGPT has been shown to have a good correlation with VFA production in the rumen (Blümmel and Ørskov, 1993; Rymer and Givens, 1998; Brown et al., 2002) the volume of gas produced during IVGPT is dependent on the stoichiometry of VFA production which in turn is dependent on the type of carbohydrate fermented. For example, the fermentation of one mole of hexose to acetate or butyrate produces 4 moles of direct gas (carbon dioxide and methane) and 3 moles of indirect carbon dioxide produced by the reaction of VFA with the bicarbonate buffer. In contrast, the fermentation of one mole of hexose to propionate, which contains an extra carbon atom, produces only 2 moles of indirect gas and no direct gas (Wolin, 1960). As a result, the fermentation of feeds with a high concentration of quickly fermentable carbohydrate and/or those that result in high propionate production is commonly associated with a lower volume of gas production compared to feeds with a higher concentration of slowly fermenting carbohydrate, such as cellulose.

Because feeds are commonly composed of a range of carbohydrates, variation in the volume of gas produced during fermentation of different carbohydrates in IVGPT means that interpretation of gas data to estimate the quantity of degraded carbohydrate is difficult. In an attempt to determine the range and quantity of carbohydrate fermented during IVPGT, proximate analysis is firstly used to measure the concentration of different carbohydrates in the feed and then, using an assumed fermentation profile, the quantity of carbohydrate fermented is estimated using multiplication factors (Herrero and Jessop, 1996; Chai et al., 2004). These multiplication factors are estimated by incubating pure or isolated substrates and measuring the volume of gas produced during IVGPT per mg of degraded sample (Table 1.2).

Unfortunately, only a few of these studies measured the microbial population of the rumen liquor prior to incubation in an attempt to standardise the inoculum (Herrero and Jessop, 1996; Jessop and Herrero, 1996). Consequently, there may be large variation in the microbial activity of inocula used in these studies, which along with the lack of a standard procedure for correction of blanks gas production and protein fermentation, limits the application of published multiplication factors and could account for the range of multiplication factors suggested for carbohydrates such as NDF and cellulose (Table 1.2).
Table 1.2. IVGPT multiplication factors for a range of carbohydrate types

<table>
<thead>
<tr>
<th>Carbohydrate type</th>
<th>ml gas / mgDM</th>
<th>ml gas / mgOM</th>
<th>Author(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non starch polysaccharide</td>
<td>0.20</td>
<td></td>
<td>Longland et al., 1995</td>
</tr>
<tr>
<td>Aqueous grass hay solubles</td>
<td>0.24</td>
<td></td>
<td>Stefanon et al., 1996</td>
</tr>
<tr>
<td>Pectin</td>
<td>0.44</td>
<td></td>
<td>Cone et al., 1997</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.37</td>
<td></td>
<td>Cone et al., 1997</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.36</td>
<td></td>
<td>Jessop and Herrero, 1996</td>
</tr>
<tr>
<td>Maize starch</td>
<td>0.35</td>
<td></td>
<td>Guzzon et al., 1997</td>
</tr>
<tr>
<td>Maize starch</td>
<td>0.34</td>
<td>0.34</td>
<td>Chai et al., 2004</td>
</tr>
<tr>
<td>Wheat starch</td>
<td>0.32</td>
<td></td>
<td>Chai et al., 2004</td>
</tr>
<tr>
<td>Barley starch</td>
<td>0.32</td>
<td></td>
<td>Chai et al., 2004</td>
</tr>
<tr>
<td>Pea starch</td>
<td>0.33</td>
<td></td>
<td>Chai et al., 2004</td>
</tr>
<tr>
<td>Potato starch</td>
<td>0.38</td>
<td></td>
<td>Chai et al., 2004</td>
</tr>
<tr>
<td>NDF</td>
<td>0.36</td>
<td></td>
<td>Stefanon et al., 1996</td>
</tr>
<tr>
<td>NDF</td>
<td>0.39</td>
<td></td>
<td>Pell and Schofield, 1993</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0.38</td>
<td></td>
<td>Cone et al., 1997</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0.44</td>
<td></td>
<td>Herrero and Jessop, 1996</td>
</tr>
</tbody>
</table>

1.2.4.3 SAMPLE PREPARATION

Although IVGPT presents the opportunity of developing more powerful rumen models to predict nutrient supply, these models are still dependent on the accurate description and analysis of feed. In common with most analytical procedures, prior to incubation in IVGPT, feeds are dried at 60°C for 24 hours and then milled to pass through 1-4mm sieves. Although this creates homogenous samples that are easily stored, analysing feeds after they have been dried and milled may not represent the characteristics of the feed in its fresh state and may mean that subsequent estimates of the rate and extent of rumen digestion may be misleading (Lowman et al., 2002).

All forms of drying that involve heat have been shown to cause morphological change in the chemical composition and structure of plant components (Van Soest, 1982) including alteration to the quantity and structure of non-structural carbohydrate (Acosta and Kothmann, 1978; Piccaglia and Galletti, 1987; Ljókel et al., 2003) and insoluble protein (Van Soest, 1982, Chamley and Viera, 1990). Drying at moderate temperatures denatures insoluble protein and decreases their rate of degradation, but at high temperatures (>100°C) carbohydrate and protein can combine to form Maillard products which are highly indigestible (Van Soest and Mason, 1991;
Deinum and Maassen, 1994) and significantly reduce the degradability of protein in the rumen (Chamley et al., 1990).

Heat drying of silage and fermented feeds also results in the loss of volatile alcohols, organic esters, amines and ammonia (Deinum and Maassen, 1994; Snyman and Joubert, 1992) which account for a large proportion of the digestible fraction of fresh feeds (Pichard and Van Soest, 1977). This has a pronounced effect on the degradation profile of immature legumes and grasses, which have a high concentration of ammonia and amines (Hoffman et al., 1993) and on the protein degradation profile of ensiled forages in which the protein is primarily composed of free amino acids and ammonia (Givens and Rulquin, 2003).

As a consequence, heat drying of feeds results in a significant reduction in the quantity of organic matter (Acosta and Koithmann, 1978) and the digestibility of available protein and carbohydrate (Broesder et al., 1992; Bonsi et al., 1995; Nagadi et al., 1998; Valentine et al., 1999). IVGPT studies have shown that dried temperate forages typically have a lower concentration and rate of fermentation of QCHO (Cone, 1998; Nagadi et al., 1998), a shorter lag (Nagadi, 1999) and a faster rate of fermentation of SCHO (Bonsi et al., 1995; Cone, 1998).

Alternative methods of sample preservation include using liquid nitrogen or freeze drying. Liquid nitrogen produces similar effects as heat drying and milling of samples (Abdalla et al., 1988; Alomar et al., 1999) but freeze dried material results in higher soluble nitrogen, lower Neutral Detergent Indigestible Nitrogen (NDIN), higher protein degradability (Abdalla et al., 1988) and higher in vitro OM digestibility (Broesder et al., 1992) compared to heat dried and milled samples. IVGPT studies have shown that freeze dried and milled maize silage produced less gas in the early stages of incubation than fresh chopped material, but after 3-4 hours are shown to exhibit a higher rate and volume of gas production (Sanderson et al., 1997; Cone, 1998). These results indicate that whilst freeze drying results in the loss and/or alteration of a proportion of the QCHO fraction, milling dried samples to 1mm may result in overestimation of the rate of carbohydrate degradation in the rumen by destroying the unique physical characteristics of each forage.

After consuming fresh material, the feed particles found in the rumen are considerably larger than those commonly found in dried and milled samples (Poppi et al., 1981). In addition to its chemical composition, the rate and
extent of feed degradation in the rumen is primarily influenced by particle size as it is this which determines the surface area available for microbial attachment and fermentation (Lowman et al., 2002). In contrast to the homogenous sample of fine particles produced by milling dried material, grinding fresh material often results in a heterogeneous sample of coarse particles that are difficult to handle, decrease the accuracy of measurements and increase the variation between samples (Lopez et al., 1995). Because fresh material is also difficult to grind, fresh samples are usually incubated whole (Nagadi, 1999), cut with scissors (Cone, 1998; Nagadi, 1999; Valentin et al., 1999) or crushed using a pestle and mortar (Nagadi, 1999) to simulate mastication. The lack of standard procedure to process fresh material and the subjective nature of the processing techniques employed means that it is extremely difficult to draw comparisons between studies using fresh material.

However, electric blenders have been successfully used to produce homogenous samples of frozen plant material for freeze drying (Alomar et al., 1999) and so a comparison of the degradation characteristics of dried and milled samples with frozen samples chopped in an electric blender may provide a clearer insight in to the effect of sample processing.

1.2.4.4 USING IVGPT TO ESTIMATE THE KINETICS OF PROTEIN DEGRADATION IN THE RUMEN

The rate of cumulative gas production during IVGPT has been shown to be highly sensitive to nitrogen availability (Raab et al., 1983; Nagadi, 1999) because when the Adenosine Tri Phosphate (ATP) released from carbohydrate degradation exceeds the maintenance requirements of the rumen microbes, a proportion is used for microbial growth which is dependent on nitrogen.

When nitrogen is unlimited, the rate of gas production and microbial protein synthesis (MPS) are directly related to the quantity of substrate fermented (Hungate, 1955, Raab et al., 1983) which in turn is directly associated with a linear decline in rumen ammonia level (Raab et al., 1983). When nitrogen is limiting, MPS decreases, a greater proportion of carbohydrate is used to fuel microbial maintenance requirements and gas production decreases. Because feed protein liberated during incubation is used in part for MPS, it has been suggested that the rate and volume of gas produced during IVGPT can also be regarded as a measure of the energy available for protein synthesis (Raab et al., 1983). This is supported by the findings of Krishnamorthy et al., (1991)
who showed that during incubation of a carbohydrate mixture (10% glucose, 40% starch, 50% cellulose) there was a positive linear relationship between MPS and cumulative gas production in the first 8 hours, after which the efficiency of MPS was dependent on the rate of fermentation of the substrate and nitrogen availability (Russel et al., 1992).

On the assumption that feed protein is highly degradable in the presence of fermentable carbohydrate, these findings offer the possibility of using cumulative gas production as an index of the total microbial growth potential of feedstuffs. Because the rate of gas production is sensitive to nitrogen availability (Nagadi, 1999), if a known quantity of dietary nitrogen is incubated in ammonia free rumen liquor, then the only nitrogen available to the microbes will come from the incubated feed. Ensuring that carbohydrate is never limiting by incubating an excessive amount of highly fermentable carbohydrate with each sample, the rate of gas production may theoretically be used as an index of protein degradation in the rumen.
1.3 AIMS AND OBJECTIVES OF THIS THESIS

1. To measure the indirect gas produced by SFA in fresh and oven-dried silages and to assess its effect on the resulting gas production profile and carbohydrate parameter estimates.

2. To study the effect of sample processing on the pattern of gas production by examining:
   
i) The particle size distribution of oven-dried and milled silages and the effect of particle size on the gas production profile and carbohydrate parameter estimates.
   ii) Comparing the gas production profile and carbohydrate parameter estimates of fresh and oven-dry silages

3. To examine the suitability of using IVGPT to assess nitrogen availability by studying the effect of fermentable nitrogen availability on the gas production profile of sucrose, glucose and maize starch.

4. To examine the potential of zeolite to remove background ammonia from rumen liquor and to study the effect of filtration through zeolite on the concentration and activity of the microbial population.

5. To compare the estimated protein degradation parameters of grass silage using IVGPT and the in situ nylon bag technique.

6. To investigate the effect of sample processing on protein degradation by comparing the estimated protein degradation parameters of fresh and dry silages using IVPGT.
CHAPTER 2

Interference of indirect gas produced by the fermentation acids of grass silage, maize silage and wholecrop wheat in an in vitro gas production technique
2.1 ABSTRACT

Volatile fatty acids (VFA) resulting from rumen fermentation are measured in the in vitro gas production technique (IVGPT) by including bicarbonate in the incubation medium and measuring the ‘indirect’ gas produced. During ensiling anaerobic fermentation of plant organic matter also produces VFA, along with lactic acid. The aim of this study was to establish whether silage fermentation acids (SFA) affected gas production profiles and subsequent carbohydrate parameter estimates of fresh and dried silages.

Results indicate that a substantial volume of SFA gas is produced during fermentation of both fresh and dried silages. SFA gas production reaches an asymptote at 30 minutes post-incubation and is 80.9% higher (P<0.001) in fresh grass silage, 42.9% higher (P<0.05) in fresh maize silage and 25.5% higher (P<0.05) in fresh wholecrop wheat compared to dried material. Failure to correct for SFA gas resulted in overestimation of quickly fermentable carbohydrate (QCHO), lag and totally fermentable carbohydrate (TCHO) concentration as well as underestimation of the slowly fermentable carbohydrate (SCHO) concentration in all silage types, fresh and dried.

Multiple linear regression equations were developed to predict the SFA gas volume produced at 30 minutes of incubation by fresh grass silage ($R^2 = 0.91$, $\text{SEE} = 0.62$, $P<0.01$), fresh maize silage ($R^2 = 0.79$, $\text{SEE} = 0.51$, $P<0.001$) and fresh wholecrop wheat ($R^2 = 0.61$, $\text{SEE} = 0.17$, $P<0.05$). Equations were also developed to predict the proportion of gas produced in the first 30 minutes of IVGPT that was derived from SFA for fresh grass silage ($R^2 = 0.95$, $\text{SEE} = 0.05$, $P<0.001$), fresh wholecrop wheat ($R^2 = 0.59$, $\text{SEE} = 0.03$, $P<0.05$), dry grass silage ($R^2 = 0.71$, $\text{SEE} = 0.13$, $P<0.05$) and dried wholecrop wheat ($R^2 = 0.59$, $\text{SEE} = 0.02$, $P<0.05$). No successful regressions were created to predict the proportion of indirect SFA gas produced by fresh or dried maize silage ($P>0.05$).

The conclusion of this study is that the interference of indirect SFA gas production is a significant problem when incubating fresh material and although this interference is reduced by pre-drying silages prior to incubation in IVGPT, indirect SFA gas still has a significant effect on the gas production profile.
Simulating microbial colonisation and digestion of feed in the rumen has long been attempted in an effort to predict the nutrient supply to ruminant animals. In vivo measurements are expensive and difficult to perform whilst in situ measurements using in sacco nylon bags to estimate rumen degradation (Mehrez and Ørskov, 1977) are unsuitable for soluble feeds, those with fine particles or those high in oil or starch as these can be washed out of the bag and not be degraded.

Because in vitro fermentation of feed results in the production of gas, the volume of gas produced should reflect the fermentation profile of feed in the rumen. This led to the development of the in vitro gas production technique (IVGPT), which simulates the rumen environment and allows estimation of the kinetics of rumen fermentation by measuring cumulative gas produced (Menke and Steingass, 1988). Microbial fermentation of feeds produces carbon dioxide, methane and short chain volatile fatty acids (VFA). Gas measured by IVGPT is produced directly from fermentation and indirectly by these VFA reacting with bicarbonate included in the incubation medium.

Anaerobic fermentation of readily fermentable plant organic matter by bacteria during the ensiling also results in the production of VFA and lactic acid, which are crucial to preservation. Deaville and Givens (1998) suggested that these silage fermentation acids (SFA) could affect the gas production profile of feeds by indirectly reacting with the bicarbonate buffer. It was subsequently recommended that all samples be pre-dried to avoid interference from this indirect gas produced by SFA.

The objective of this study was to establish the quantitative effect of SFA on observed gas production profiles and estimated carbohydrate parameters of fresh and dried grass silage, maize silage and wholecrop wheat. The aims were to (1) measure the volume of indirect gas produced by fresh and dried silages, (2) to assess what effect this indirect SFA gas has on the estimated degradation parameters of these forages during IVGPT and, (3) to develop equations to predict the volume and/or proportion of indirect SFA gas produced during IVGPT.
2.3 MATERIAL AND METHODS

2.3.1 EXPERIMENTAL TREATMENTS

EXPERIMENT 1

This experiment was designed to establish the quantity and rate of production of indirect gas to SFA by incubating samples in anaerobic buffer medium (ABM) and measuring cumulative gas produced in the absence of rumen microbes.

EXPERIMENT 2

Having determined the level of indirect gas attributable to SFA from experiment 1, this experiment was designed to establish how indirect gas produced by SFA affected estimation of carbohydrate fermentation parameters using IVGPT and to develop multiple linear equations to predict indirect gas production from SFA during IVGPT.

2.3.2 PROXIMATE ANALYSIS

Samples of 11 grass silages made from predominantly perennial ryegrass swards, 13 maize silages, and 11 wholecrop wheat samples, all cut in 2003, were obtained from commercial farms through-out the United Kingdom (UK) by Bioparametrics Ltd, an Edinburgh based analytical company interested in developing IVGPT for commercial applications.

The DM content of each forage sample was measured by drying approximately 100g of fresh material in a 60°C oven for 24 hours. The dried samples were then milled through a 1mm sieve using a Retsch® mill, analysed for NDF (Van Soest et al., 1991) and ADF using ANKOM F57® fibre bags and crude protein (CP) using the standard Kjedahl technique. See Appendix A.1 for details of the methodology used. The lactic acid (g/kgDM), total volatile fatty acid (VFA) (g/kgDM) content and pH of each of the silages was estimated by NIRS (Foss NIRSystem 6500) using calibrations developed by the Forage Analysis Assurance FAA (FAA) group.

To simulate mastication, fresh forage samples were frozen overnight and then approximately 15g of the frozen material was chopped in a Moulinex Ovatio 3 Duo blender at 1450rpm for 15 seconds.
2.3.3 ESTIMATING THE INDIRECT SFA GAS PRODUCED DURING IVGPT

After simulated mastication, the fresh samples were allowed to defrost at room temperature for twenty minutes. The equivalent of 220 mg DM of dry and fresh silage were weighed in duplicate into 100 ml glass syringes and placed in the fridge until the morning of incubation. Three additional syringes were also prepared and placed in the fridge but contained no samples.

On the morning of inoculation and prior to preparing the anaerobic buffer medium (ABM), all the syringes were placed in a 39°C water bath to reach temperature.

ABM was prepared according to Menke and Steingass (1988), placed in a 39°C water bath and saturated with CO₂ gas. Using an automatic dispensing pump, 30ml of ABM was added to each fermentation syringe before it was returned to the water bath. The cumulative gas production for each syringe was read every 15 minutes for the first 2 hours and thereafter at 2.5, 3 and 3.5 h. Syringes were manually shaken at each reading.

2.3.4 ESTIMATING THE RUMEN FERMENTATION PROFILE OF SILAGE DURING IVGPT

After simulated mastication, the fresh samples were allowed to defrost at room temperature for twenty minutes. The equivalent of 220 mg DM of dry and fresh silage were weighed in duplicate into 100 ml glass syringes and placed in the fridge until the morning of incubation. Three additional syringes were also created and placed in the fridge but contained no samples.

On the morning of inoculation and prior to collecting the rumen liquor, the syringes containing the samples, along with ‘blanks’ containing no substrate, were placed in a 39°C water bath.

Rumen liquor was collected from sheep kept outside during summer and grazing predominantly perennial ryegrass with no access to supplemental feeds. One sheep was used for each gas production run. On the morning of inoculation, individual sheep were collected from the pasture at approximately 09:30, slaughtered and the rumen and reticulum removed and placed in a thermos box to keep warm whilst travelling back to the laboratory. At the laboratory the rumen contents were strained through two
layers of 250 μm netting and three layers of muslin, and then stored in a thermos flask gassed with CO₂.

To ensure an adequate microbial population is incubated with each sample and to enable comparison of samples in different gas production runs, microbial activity of the rumen liquor was assessed by measuring its absorbance at 600 nm (Nagadi et al., 2000). The dilution ratio with anaerobic medium was then calculated to ensure that the inoculum had an absorbance at 600 nm of 0.111 - 0.134, thereby ensuring that microbial activity among the IVGPT runs was similar.

The appropriate volume of ABM (Menke and Steingass, 1988) was prepared, placed in a 39°C water bath and saturated with CO₂. When the medium reached 39°C, rumen liquor was added and the inoculum was gassed with CO₂ for a further 5 minutes. Using an automatic dispensing pump, 30 ml of inoculum was added to each fermentation syringe before it was returned to the water bath. Measurement of in vitro gas production were performed as described by Jessop and Herrero (1996), with cumulative gas production in each syringe read every half hour for the first 3 hours, then hourly until 6 hours and thereafter at 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 56, 64, and 72, 80, 88 and 96h. Syringes were manually shaken at each reading.

2.3.5 INTERPRETATION OF GAS PRODUCTION DATA

EXPERIMENT 1

The cumulative gas production of the samples incubated in ABM was analysed using a non-linear procedure by fitting the model: \( \text{Gas} = B(1-\exp^{-C_b \times \text{time}}) \) where B represents the asymptote of gas production and Cb the fractional rate (h⁻¹) of gas production.

The cumulative gas volumes were first corrected to fermentation of 220mgDM and then corrected for any background effects such as atmospheric pressure by fitting the gas production profiles of the ‘blanks’ to the model: \( \text{Gas} = B(1-\exp^{-C_b \times \text{time}}) \) and subtracting the mean gas production of the blanks from all gas profiles. The adjusted cumulative gas volumes of each sample was then analysed using the same model.
**EXPERIMENT 2**

The fermentation profile of samples incubated with rumen microbes was determined by applying the analytical procedure described in Appendix A1.4. These describe forage fermentation in terms of the quantity and rate of quickly fermented carbohydrate (QCHO) fermentation and then after a lag, the quantity and rate of slowly fermented carbohydrate (SCHO).

To compare the effect of indirect SFA gas, the procedure was repeated having subtracted the volume of indirect gas produced in the first 30 minutes of incubation in ABM (*Experiment 1*) from all the gas production measurements taken during incubation of samples with rumen microbes.

**2.3.6 CALCULATING THE PROPORTION OF IVGPT GAS ATTRIBUTED TO INDIRECT SFA GAS**

To calculate the proportion of gas produced during incubation with rumen microbes that can be attributed to the indirect gas produced by SFA, the volume of indirect gas produced in the first 30 minutes during incubation of samples in ABM (IVGPTB) was divided by the volume of gas produced in the first 30 minutes during incubation of samples with rumen microbes (IVGPT):

\[
\text{Proportion (fraction)} = \frac{\text{IVGPTB} @ 30 \text{ minutes (ml)}}{\text{IVGPT} @ 30 \text{ minutes (ml)}}
\]

**2.3.7 STATISTICAL ANALYSIS**

Using the MINITAB statistical package (2000), paired T-tests were used to compare the cumulative gas production and degradation parameters of the dried and fresh forages. Analysis of variance (ANOVA) using a general linear model (GLM) was used to compare the difference between different forage types. Multiple linear regression was used to predict the volume and proportion of indirect gas attributable to SFA that is produced during incubation of silage samples using IVGPT using DM, lactic acid, VFA and pH as independent prediction variables. The independent variables used for each forage type was selected using the ‘best subset’ function within the statistical software (Minitab, 2000). Outliers were removed using the ‘unusual observation function’ within the Minitab (2000), which removes observations with a standardized residual greater than 2.
2.4 RESULTS

2.4.1 PROXIMATE ANALYSIS

The proximate analyses results for the forage samples studied are presented in Table 2.1.

Table 2.1 Proximate Analyses of grass silage, maize silage and wholecrop Wheat. Standard deviation values are shown in brackets.

<table>
<thead>
<tr>
<th>Type</th>
<th>Grass Silage</th>
<th>Maize Silage</th>
<th>Wholecrop Wheat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>11</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>DM (g/kg)</td>
<td>298.0 (87.2)</td>
<td>294.6 (51.7)</td>
<td>487.6 (151)</td>
</tr>
<tr>
<td>ADF (g/kgDM)</td>
<td>311.2 (43.3)</td>
<td>270.2 (25.6)</td>
<td>351.9 (76.7)</td>
</tr>
<tr>
<td>NDF (g/kgDM)</td>
<td>468.4 (61.2)</td>
<td>412.0 (39.8)</td>
<td>465.4 (77.5)</td>
</tr>
<tr>
<td>CP (g/kgDM)</td>
<td>122.4 (22.83)</td>
<td>69.9 (8.96)</td>
<td>80.7 (24.70)</td>
</tr>
<tr>
<td>Lactic Acid (g/kgDM)</td>
<td>84.23 (28.20)</td>
<td>26.48 (18.81)</td>
<td>9.50 (12.23)</td>
</tr>
<tr>
<td>VFAcid (g/kgDM)</td>
<td>34.30 (34.1)</td>
<td>31.68 (17.88)</td>
<td>62.95 (24.5)</td>
</tr>
<tr>
<td>pH</td>
<td>3.99 (0.47)</td>
<td>4.91 (0.68)</td>
<td>5.69 (1.18)</td>
</tr>
</tbody>
</table>

2.4.2 EXPERIMENT 1 – MEASURING THE INDIRECT GAS PRODUCED BY THE SILAGE FERMENTATION ACIDS (SFA) OF FRESH AND DRIED GRASS SILAGE, MAIZE SILAGE AND WHOLECROP WHEAT DURING IVGPT IN THE ABSENCE OF RUMEN MICROBES

The cumulative gas produced during incubation of dried and fresh grass silage, maize silage and wholecrop wheat in ABM is presented in Table 2.2 and 2.3 and illustrated in Fig 2.1 and Fig 2.2.

The incubation of all dried and fresh forages in ABM produced a substantial volume of gas in the first 30 minutes, probably due to SFA in the silage reacting with bicarbonate buffer in the anaerobic medium (Table 2.2). Although a significant quantity of gas was still produced after 45 minutes (P<0.05) in all the silage types, this extra gas production was within the accuracy of the technique (±0.25ml) and therefore did not differ.

The quantity and rate of indirect gas production was significantly higher in the fresh material of all comparable forage types. In comparison to dried material, fresh grass silage produced 80.9% more gas (P<0.001) at a 38%
higher rate, fresh maize silage produced 42.9% more gas (P<0.05) at a 19.9% higher rate and fresh wholecrop wheat produced 25.5% more gas (P<0.05) at an 18.5% higher rate.

The highest level and rate of gas production was from grass silage which produced significantly more gas and at a quicker rate than maize silage and wholecrop wheat in both fresh and dried material (P<0.05) (Table 2.3). The volume and rate of gas produced by maize silage and wholecrop wheat was not significantly different for either fresh or dried material (P>0.05). Grass silage also exhibited the largest range of gas produced by both fresh (±1.32ml) and dried (±0.77ml) material, followed by maize silage (±1.02ml; ±0.23ml) and finally wholecrop wheat which had the smallest range (±0.41ml; ±0.18ml) (Table 2.3).

**Table 2.2** Estimated gas production parameters of 220mgDM of fresh and dried grass silage (11 samples), maize silage (13 samples) and wholecrop wheat (11 samples) incubated in anaerobic medium. Each sample was incubated in duplicate (n=2). Standard deviation values are shown in brackets. For each forage type, means with different superscripts within the same column are significantly different (P<0.05).

<table>
<thead>
<tr>
<th>Type</th>
<th>Parameters(^\text{c})</th>
<th>Dry &amp; Milled</th>
<th>Fresh &amp; Chopped</th>
<th>Sig.</th>
<th>Diff. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass Silage</td>
<td>b (ml)</td>
<td>2.42 (0.71)</td>
<td>4.38 (1.32)</td>
<td>***</td>
<td>+80.9</td>
</tr>
<tr>
<td></td>
<td>cb (/h)</td>
<td>6.94 (1.52)</td>
<td>9.58 (1.59)</td>
<td>**</td>
<td>+38.0</td>
</tr>
<tr>
<td></td>
<td>SFA Vol @0.50h (ml)</td>
<td>2.36 (0.77)(^a)</td>
<td>4.34 (1.32)(^a)</td>
<td>***</td>
<td>+83.9</td>
</tr>
<tr>
<td></td>
<td>SFA Vol @0.75h (ml)</td>
<td>2.42 (0.76)(^b)</td>
<td>4.38 (1.32)(^b)</td>
<td>***</td>
<td>+80.9</td>
</tr>
<tr>
<td>Maize Silage</td>
<td>b (ml)</td>
<td>1.56 (0.23)</td>
<td>2.23 (1.02)</td>
<td>*</td>
<td>+42.9</td>
</tr>
<tr>
<td></td>
<td>cb (/h)</td>
<td>5.21 (0.24)</td>
<td>6.25 (1.44)</td>
<td>*</td>
<td>+19.9</td>
</tr>
<tr>
<td></td>
<td>SFA Vol @0.50h (ml)</td>
<td>1.43 (0.21)(^a)</td>
<td>2.00 (0.95)(^a)</td>
<td>*</td>
<td>+39.9</td>
</tr>
<tr>
<td></td>
<td>SFA Vol @0.75h (ml)</td>
<td>1.54 (0.23)(^b)</td>
<td>2.02 (0.92)(^b)</td>
<td>*</td>
<td>+31.2</td>
</tr>
<tr>
<td>Wholecrop</td>
<td>b (ml)</td>
<td>1.53 (0.18)</td>
<td>1.92 (0.41)</td>
<td>*</td>
<td>+25.5</td>
</tr>
<tr>
<td>Wheat</td>
<td>cb (/h)</td>
<td>5.42 (0.73)</td>
<td>6.65 (1.25)</td>
<td>*</td>
<td>+18.5</td>
</tr>
<tr>
<td></td>
<td>SFA Vol @0.50h (ml)</td>
<td>1.43 (0.19)(^a)</td>
<td>1.85 (0.44)(^a)</td>
<td>*</td>
<td>+29.4</td>
</tr>
<tr>
<td></td>
<td>SFA Vol @0.75h (ml)</td>
<td>1.50 (0.18)(^b)</td>
<td>1.91 (0.42)(^b)</td>
<td>*</td>
<td>+22.7</td>
</tr>
</tbody>
</table>

\(^{\text{c}}\)B and Cb are parameters obtained by fitting the gas production data to the equation: \(\text{Gas} = B/(1-\exp(-C b^{\text{time}}))\) where B represents the asymptote of gas production and C the fractional rate (h\(^{-1}\)) of gas production.
Fig 2.1 Mean cumulative gas production of dry and milled (white) and fresh and chopped (black) grass silage (squares) \( (n=11) \), maize silage (triangle) \( (n=13) \) and wholecrop wheat (circle) \( (n=11) \) incubated in duplicate in buffer medium and fitted to the equation \( \text{Gas} = B(1-e^{-\text{Cp time}}) \) where B represents the asymptote of gas production and \( \text{C}_b \) the fractional rate \( (\text{h}^{-1}) \) of gas production.

Table 2.3 Estimated gas production parameters of 220mgDM of fresh and dried grass silage (11 samples), maize silage (13 samples) and wholecrop wheat (11 samples) incubated in anaerobic medium. Each sample was incubated in duplicate \( (n=2) \). Standard deviation values are shown in brackets. For each forage type, means with different superscripts within the same row are significantly different \( (P<0.05) \).

<table>
<thead>
<tr>
<th>Type</th>
<th>Parameters(^i)</th>
<th>Grass silage</th>
<th>Maize silage</th>
<th>Wholecrop Wheat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry &amp;</td>
<td>b (ml)</td>
<td>2.42 (0.71)(^a)</td>
<td>1.56 (0.23)(^b)</td>
<td>1.53 (0.18)(^b)</td>
</tr>
<tr>
<td></td>
<td>cb (l/h)</td>
<td>6.94 (1.52)(^a)</td>
<td>5.21 (0.24)(^b)</td>
<td>5.42 (0.73)(^b)</td>
</tr>
<tr>
<td>Milled</td>
<td>SFA Vol @0.50h (ml)</td>
<td>2.36 (0.77)(^a)</td>
<td>1.43 (0.21)(^b)</td>
<td>1.43 (0.19)(^b)</td>
</tr>
<tr>
<td></td>
<td>SFA Vol @0.75h (ml)</td>
<td>2.42 (0.76)(^a)</td>
<td>1.54 (0.23)(^b)</td>
<td>1.50 (0.18)(^b)</td>
</tr>
<tr>
<td>Fresh &amp;</td>
<td>b (ml)</td>
<td>4.38 (1.32)(^a)</td>
<td>2.23 (1.02)(^b)</td>
<td>1.92 (0.41)(^b)</td>
</tr>
<tr>
<td>Chopped</td>
<td>cb (l/h)</td>
<td>9.58 (1.59)(^a)</td>
<td>6.25 (1.44)(^b)</td>
<td>6.65 (1.25)(^b)</td>
</tr>
<tr>
<td></td>
<td>SFA Vol @0.50h (ml)</td>
<td>4.34 (1.32)(^a)</td>
<td>2.00 (0.95)(^b)</td>
<td>1.85 (0.44)(^b)</td>
</tr>
<tr>
<td></td>
<td>SFA Vol @0.75h (ml)</td>
<td>4.38 (1.32)(^a)</td>
<td>2.02 (0.92)(^b)</td>
<td>1.91 (0.42)(^b)</td>
</tr>
</tbody>
</table>

\(^i\)B and Cb are parameters obtained by fitting the gas production data to the equation: \( \text{Gas} = B(1-e^{-\text{Cp time}}) \) where B represents the asymptote of gas production and \( \text{C}_b \) the fractional rate \( (\text{h}^{-1}) \) of gas production.
Fig 2.2 Comparison of the gas produced in the first 30 minutes of incubation in anaerobic buffer medium by dry and milled (shaded) and fresh and chopped (white) grass silage \((n=11)\), maize silage \((n=13)\) and wholecrop wheat \((n=11)\). Each sample was incubated in duplicate in buffer medium and the cumulative gas production fitted to the equation \(\text{Gas} = B(1-\exp(-C\times\text{time}))\) where \(B\) represents the asymptote of gas production and \(C\) the fractional rate \((\text{h}^{-1})\) of gas production. Standard deviation values are illustrated using \(Y\)-bars.

### 2.4.3 EXPERIMENT 2 – EFFECT OF SFA GAS PRODUCTION ON THE CARBOHYDRATE DEGRADATION PARAMETERS OF FRESH AND DRIED GRASS SILAGE, MAIZE SILAGE AND WHOLECROP WHEAT DURING IVGPT

The effect SFA gas production on the cumulative gas production profiles of dried and fresh grass silage, maize silage and wholecrop wheat during IVGPT is presented in Table 2.4, Table 2.5 and Table 2.6 and illustrated in Fig 2.3, Fig 2.4 and Fig 2.5.

Failure to account for indirect gas produced by SFA results in the overestimation of QCHO, time lag and TCHO and underestimation of the concentration of SCHO in all the forages and in both fresh and dried material. However, the degradation parameter that is most effected by failure to correct for indirect SFA gas production is QCHO which is overestimated by 26.8% \((P<0.001)\) in fresh grass silage (Table 2.2), 25.8% \((P<0.001)\) in fresh maize silage (Table 2.3), 25.1% \((P<0.001)\) in fresh wholecrop wheat (Table 2.4), 19.2% \((P<0.001)\) in dried grass silage (Table 2.2), 18.6% \((P<0.001)\) in dried maize silage (Table 2.3) and 19.7% \((P<0.001)\) in dried wholecrop wheat (Table 2.4).
Table 2.4 The effect of SFA gas production on the estimated gas production degradability parameters of 220mg DM of dried and fresh grass silage incubated in ovine ruminal fluid. Each value is a mean of 11 samples incubated in duplicate (n=2). Standard deviation values are shown in brackets.

<table>
<thead>
<tr>
<th>Type</th>
<th>Uncorrected for SFA gas</th>
<th>Corrected for SFA gas</th>
<th>Sig.</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QCHO (g/kgDM)</td>
<td>252.6 (82.5)</td>
<td>204.2 (67.7)</td>
<td>***</td>
<td>-19.2</td>
</tr>
<tr>
<td>Ca (h)</td>
<td>0.203 (0.005)</td>
<td>0.200 (0.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry &amp; SCHO (g/kgDM)</td>
<td>311.6 (56.8)</td>
<td>338.4 (57.0)</td>
<td>***</td>
<td>+8.6</td>
</tr>
<tr>
<td>Milled Cb (h)</td>
<td>0.087 (0.025)</td>
<td>0.088 (0.025)</td>
<td>*</td>
<td>+0.92</td>
</tr>
<tr>
<td>Lag (h)</td>
<td>6.37 (0.98)</td>
<td>5.64 (1.34)</td>
<td>***</td>
<td>-11.5</td>
</tr>
<tr>
<td>TCHO (g/kgDM)</td>
<td>514.5 (12.8)</td>
<td>501.4 (12.5)</td>
<td>***</td>
<td>-2.5</td>
</tr>
<tr>
<td>QCHO (g/kgDM)</td>
<td>382.1 (86.2)</td>
<td>279.7 (66.7)</td>
<td>***</td>
<td>-26.8</td>
</tr>
<tr>
<td>Ca (h)</td>
<td>0.209 (0.003)</td>
<td>0.202 (0.004)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh &amp; SCHO (g/kgDM)</td>
<td>322.8 (72.0)</td>
<td>377 (71.7)</td>
<td>***</td>
<td>+16.8</td>
</tr>
<tr>
<td>Chop’d Cb (h)</td>
<td>0.072 (0.016)</td>
<td>0.076 (0.015)</td>
<td>***</td>
<td>+5.1</td>
</tr>
<tr>
<td>Lag (h)</td>
<td>6.63 (1.50)</td>
<td>5.18 (1.64)</td>
<td>***</td>
<td>-21.9</td>
</tr>
<tr>
<td>TCHO (g/kgDM)</td>
<td>633.4 (17.1)</td>
<td>601.3 (17.6)</td>
<td>***</td>
<td>-5.1</td>
</tr>
</tbody>
</table>

NS P>0.05  *P<0.05  **P<0.01  ***P<0.001

Fig 2.3 Cumulative gas production of dried and milled (triangle) and fresh and chopped (square) grass silage corrected (white) and uncorrected (black) for indirect gas produced by SFA when incubated in ovine rumen liquor during IVGPT. Each cumulative gas curve is the mean of 11 samples incubated in duplicate and fitted to the equation: \( \text{Gas} = A(1-\exp(-\text{cd})) + B(1-\exp(-\text{c}(\text{t-Lag}))) \times \exp(5(\text{t-Lag}))/\left(1+\exp(5(\text{t-Lag}))\right) \) modified from Jessop and Herrero (1996). [see text for interpretation of parameters].
Table 2.5 The effect of SFA gas production on the estimated gas production degradability parameters of 220mg DM of dried and fresh Maize silage incubated in ovine ruminal fluid. Each value is a mean of 11 samples incubated in duplicate (n=2). Standard deviation values are shown in brackets.

<table>
<thead>
<tr>
<th>Type</th>
<th>Uncorrected for SFA gas</th>
<th>Corrected for SFA gas</th>
<th>Sig.</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>QCHO (g/kgDM)</td>
<td>136.3 (54.7)</td>
<td>110.9 (44.3)</td>
<td>***</td>
<td>-18.6</td>
</tr>
<tr>
<td>Ca (/h)</td>
<td>0.201 (0.002)</td>
<td>0.201 (0.002)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry &amp; SCHO (g/kgDM)</td>
<td>555.7 (56.8)</td>
<td>571.2 (49.8)</td>
<td>***</td>
<td>+2.8</td>
</tr>
<tr>
<td>Cb (/h)</td>
<td>0.083 (0.013)</td>
<td>0.082 (0.013)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Milled Lag (h)</td>
<td>5.38 (1.10)</td>
<td>5.12 (1.05)</td>
<td>***</td>
<td>-4.8</td>
</tr>
<tr>
<td>TCHO (g/kgDM)</td>
<td>664.5 (8.7)</td>
<td>659.8 (8.6)</td>
<td>***</td>
<td>-0.71</td>
</tr>
</tbody>
</table>

QCHO (g/kgDM) | 162.7 (90.9)            | 120.8 (57.1)          | ***  | -25.8          |
| Ca (/h)       | 0.201 (0.002)           | 0.200 (0.00)          |      |                |
| Fresh & SCHO (g/kgDM) | 585.1 (87.1)      | 609.5 (71.8)          | ***  | +4.2           |
| Chop’d Cb (/h) | 0.0712 (0.010)          | 0.0721 (0.010)        | NS   |                |
| Lag (h)       | 5.42 (0.91)             | 5.05 (0.95)           | ***  | -6.8           |
| TCHO (g/kgDM) | 715.4 (11.1)            | 705.9 (11.4)          | ***  | -1.3           |

NS P>0.05 *P<0.05 **P<0.01 ***P<0.001

Fig 2.4 Cumulative gas production of dried and milled (triangle) and fresh and chopped (square) maize silage corrected (white) and uncorrected (black) for indirect gas produced by SFA when incubated in ovine rumen liquor during IVGPT. Each cumulative gas curve is the mean of 11 samples incubated in duplicate and fitted to the equation: \( \text{Gas} = A(1-\exp(-c_1t))+B(1-\exp(-c_2(t-Lag)))\ast\exp(5(t-Lag))/(1+\exp(5(t-Lag))) \) modified from Jessop and Herrero (1996). [see text for interpretation of parameters].
Table 2.6 The effect of SFA gas production on the estimated gas production degradability parameters of 220mg DM of dried and fresh wholecrop wheat incubated in ovine ruminal fluid. Each value is a mean of 11 samples incubated in duplicate (n=2). Standard deviation values are shown in brackets.

<table>
<thead>
<tr>
<th>Type</th>
<th>Uncorrected for SFA gas</th>
<th>Corrected for SFA gas</th>
<th>Sig.</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QCHO (g/kgDM)</td>
<td>114.3 (45)</td>
<td>91.8 (36.6)</td>
<td>***</td>
<td>-19.7</td>
</tr>
<tr>
<td>Ca (h)</td>
<td>0.2007 (0.0007)</td>
<td>0.2000 (0.0000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry &amp; Milled</td>
<td>SCHO (g/kgDM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cb (h)</td>
<td>0.0612 (0.0059)</td>
<td>0.0622 (0.0061)</td>
<td>**</td>
<td>+1.6</td>
</tr>
<tr>
<td>Lag (h)</td>
<td>5.63 (0.64)</td>
<td>5.34 (0.59)</td>
<td>*</td>
<td>-5.2</td>
</tr>
<tr>
<td>TCHO (g/kgDM)</td>
<td>516.1 (38.1)</td>
<td>510.5 (37.9)</td>
<td>***</td>
<td>-1.1</td>
</tr>
<tr>
<td>Fresh &amp; Chop’d</td>
<td>SCHO (g/kgDM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cb (h)</td>
<td>0.0567 (0.0049)</td>
<td>0.0573 (0.0047)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Lag (h)</td>
<td>5.74 (0.54)</td>
<td>5.37 (0.59)</td>
<td>**</td>
<td>-6.4</td>
</tr>
<tr>
<td>TCHO (g/kgDM)</td>
<td>593.7 (44.3)</td>
<td>586.6 (43.8)</td>
<td>***</td>
<td>-1.2</td>
</tr>
</tbody>
</table>

NS P>0.05  *P<0.05  **P<0.01  ***P<0.001

Fig 2.5 Cumulative gas production of dried and milled (triangle) and fresh and chopped (square) wholecrop wheat corrected (white) and uncorrected (black) for indirect gas produced by SFA when incubated in ovine rumen liquor during IVGPT. Each cumulative gas curve is the mean of 11 samples incubated in duplicate and fitted to the equation: $Gas = A(1-exp(-c(t)))+B(1-exp(-c(t-Lag)))*exp(5(t-Lag))/(1+exp(5(t-Lag)))$ modified from Jessop and Herrero (1996). [see text for interpretation of parameters].
Before correcting for indirect SFA gas, the fresh material produced significantly more gas during the first 30 minutes of incubation in ovine rumen liquor with grass silage producing 128.5% more (P<0.001), maize silage 77.5% more gas (P<0.05) and wholecrop wheat 52.1% more gas (P<0.05) compared to dried material (Table 2.7). The proportion of gas produced in the first 30 minutes of IVGPT attributed to SFA gas production was significantly lower in dried and fresh grass silage (68%; 60%) compared to maize silage (94%; 92%) and wholecrop wheat (96%; 93%) (P<0.05), which were not significantly different (P>0.05) (Table 2.8). The proportion of SFA gas produced in the first 30 minutes of IVGPT was 11.8% less in fresh grass silage (P<0.01) compared to dried material, whilst the proportion in maize silage and wholecrop wheat was not significantly different between fresh and dried material (P>0.05). Consequently after correcting for indirect SFA gas, the volume of gas produced in the first 30 minutes of IVGPT was not significantly different between fresh and dried maize silage or wholecrop wheat, but was 195.6% higher in fresh grass silage compared to dried material (P<0.001) (Table 2.7).

Table 2.7 Quantitative assessment of the indirect SFA gas produced by 220mgDM fresh and dried grass silage (n=11), maize silage (n=13) and wholecrop wheat (n=11) incubated in anaerobic medium (SFA) and ovine rumen liquor (GP). Each sample was incubated in duplicate (n=2). Standard deviation values are shown in brackets.

<table>
<thead>
<tr>
<th>Type</th>
<th>Parameters</th>
<th>Dry &amp; Milled</th>
<th>Fresh &amp; Chopped</th>
<th>Sig.</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass</td>
<td>SFA Vol @0.50h (ml)</td>
<td>2.36 (0.77)</td>
<td>4.34 (1.32)</td>
<td>***</td>
<td>+83.9</td>
</tr>
<tr>
<td>Silage</td>
<td>GP Vol @0.50h (ml)</td>
<td>3.19 (0.92)</td>
<td>7.29 (1.04)</td>
<td>***</td>
<td>+128.5</td>
</tr>
<tr>
<td></td>
<td>SFA Proportion @0.5h</td>
<td>0.68 (0.20)</td>
<td>0.60 (0.19)</td>
<td>**</td>
<td>-11.8</td>
</tr>
<tr>
<td></td>
<td>Corrected GP @0.5h (ml)</td>
<td>1.14 (0.88)</td>
<td>3.38 (1.18)</td>
<td>***</td>
<td>+196.5</td>
</tr>
<tr>
<td>Maize</td>
<td>SFA Vol @0.50h (ml)</td>
<td>1.43 (0.21)</td>
<td>2.00 (0.95)</td>
<td>*</td>
<td>+39.9</td>
</tr>
<tr>
<td>Silage</td>
<td>GP Vol @0.50h (ml)</td>
<td>1.11 (0.65)</td>
<td>1.97 (1.69)</td>
<td>*</td>
<td>+77.5</td>
</tr>
<tr>
<td></td>
<td>SFA Proportion @0.5h</td>
<td>0.94 (0.09)</td>
<td>0.92 (0.12)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Corrected GP @0.5h (ml)</td>
<td>0.14 (0.22)</td>
<td>0.24 (0.48)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>W/crop</td>
<td>SFA Vol @0.50h (ml)</td>
<td>1.43 (0.19)</td>
<td>1.85 (0.44)</td>
<td>*</td>
<td>+29.4</td>
</tr>
<tr>
<td>Wheat</td>
<td>GP Vol @0.50h (ml)</td>
<td>0.94 (0.38)</td>
<td>1.43 (0.82)</td>
<td>*</td>
<td>+52.1</td>
</tr>
<tr>
<td></td>
<td>SFA Proportion @0.5h</td>
<td>0.96 (0.07)</td>
<td>0.93 (0.12)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Corrected GP @0.5h (ml)</td>
<td>0.05 (0.11)</td>
<td>0.11 (0.25)</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

NS P>0.05  *P<0.05  **P<0.01  ***P<0.001
Table 2.8 Quantitative assessment of the indirect SFA gas produced by 220mgDM fresh and dried grass silage (n=11), maize silage (n=13) and wholecrop wheat (n=11) incubated in anaerobic medium (SFA) and ovine rumen liquor (GP). Each sample was incubated in duplicate (n=2). For each forage type, means with different superscripts within the same row are significantly different (P<0.05).

<table>
<thead>
<tr>
<th>Type</th>
<th>Parameters</th>
<th>Grass silage</th>
<th>Maize silage</th>
<th>Wholecrop Wheat</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFA Vol @0.50h (ml)</td>
<td>2.36 (0.77)a</td>
<td>1.43 (0.21)b</td>
<td>1.43 (0.19)b</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Dry &amp; GP Vol @0.50h (ml)</td>
<td>3.19 (0.92)a</td>
<td>1.11 (0.65)b</td>
<td>0.94 (0.38)b</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Milled SFA Proportion @0.5h</td>
<td>0.68 (0.20)a</td>
<td>0.94 (0.09)b</td>
<td>0.96 (0.07)b</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Corrected GP @0.5h (ml)</td>
<td>1.14 (0.88)a</td>
<td>0.14 (0.22)b</td>
<td>0.05 (0.11)b</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Fresh &amp; GP Vol @0.50h (ml)</td>
<td>4.34 (1.32)a</td>
<td>2.00 (0.95)b</td>
<td>1.85 (0.44)b</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Chop’d SFA Proportion @0.5h</td>
<td>7.29 (1.04)a</td>
<td>1.97 (1.69)b</td>
<td>1.43 (0.82)b</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Corrected GP @0.5h (ml)</td>
<td>0.6 (0.19)a</td>
<td>0.92 (0.12)b</td>
<td>0.93 (0.12)b</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>Corrected GP @0.5h (ml)</td>
<td>3.38 (1.18)a</td>
<td>0.24 (0.48)b</td>
<td>0.11 (0.25)b</td>
<td>***</td>
<td></td>
</tr>
</tbody>
</table>

NS P>0.05  *P<0.05  **P<0.01  ***P<0.001

2.4.4 PREDICTING INDIRECT SFA GAS PRODUCTION DURING IVGPT

The volume of indirect SFA gas produced during IVGPT can be predicted by multiple linear regression for fresh grass silage using the independent variables of DM, lactic acid and VFA (R² = 0.91, SEE = 0.62, P<0.01), for fresh maize silage using DM and VFA (R² = 0.79, SEE = 0.51, P<0.001) and for fresh wholecrop wheat using VFA (R² = 0.61, SEE = 0.17, P<0.05) (Table 2.9).

The proportion of gas produced in the first 30 minutes of IVGPT that is attribute to indirect SFA gas production can be predicted by multiple linear regression for fresh grass silage using the independent variables of DM and lactic acid (R² = 0.95, SEE = 0.05, P<0.001), for dried grass silage using DM and lactic acid (R² = 0.71, SEE = 0.13, P<0.05) and for fresh wholecrop wheat (R² = 0.59, SEE = 0.03, P<0.05) and dried wholecrop wheat (R² = 0.59, SEE = 0.02, P<0.05) and using VFA. The proportion of SFA attributable gas produced by dry and fresh maize silage during IVGPT could not be predicted using the current proximate analysis (P>0.05) (Table 2.10).
Table 2.9 Multiple linear regression equations to predict the volume of SFA gas produced by 220mgDM of fresh & chopped grass silage, maize silage and wholecrop in the first 30 minutes of IVGPT in ovine rumen liquor.

<table>
<thead>
<tr>
<th>Type</th>
<th>n</th>
<th>Equation</th>
<th>$R^2$</th>
<th>SEE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass Silage</td>
<td>10</td>
<td>2.44 – 0.00474 DM (g/kg) + 0.041 Lactic Acid (g/kgDM) +0.0104 VFA (g/kgDM)</td>
<td>0.907</td>
<td>0.619</td>
<td>**</td>
</tr>
<tr>
<td>Maize Silage</td>
<td>12</td>
<td>1.8– 0.00547 DM (g/kg) + 0.0613 VFA (g/kgDM)</td>
<td>0.796</td>
<td>0.5077</td>
<td>***</td>
</tr>
<tr>
<td>W/crop Wheat</td>
<td>9</td>
<td>1.2331 + 0.0078 VFA (g/kgDM)</td>
<td>0.607</td>
<td>0.171</td>
<td>*</td>
</tr>
</tbody>
</table>

NS P>0.05  *P<0.05  **P<0.01  ***P<0.001

Table 2.10 Multiple linear regression equations to predict the proportion of gas produced in the first 30 minutes of IVGPT that can be attributed to SFA when incubating 220mgDM of dry and fresh grass silage, maize silage and wholecrop wheat in ovine rumen liquor using IVGPT.

<table>
<thead>
<tr>
<th>Type</th>
<th>n</th>
<th>Equation</th>
<th>$R^2$</th>
<th>SEE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass Silage</td>
<td>9</td>
<td>1.2614 + -0.0019 DM (g/kg) + 0.0011 VFA (g/kgDM)</td>
<td>0.709</td>
<td>0.125</td>
<td>*</td>
</tr>
<tr>
<td>Dry &amp; Milled</td>
<td>Maize Silage</td>
<td>5</td>
<td>0.9992 + -0.0045 VFA (g/kgDM)</td>
<td>0.679</td>
<td>0.0805</td>
</tr>
<tr>
<td></td>
<td>W/crop Wheat</td>
<td>7</td>
<td>0.8975 + 0.0011 VFA (g/kgDM)</td>
<td>0.589</td>
<td>0.0243</td>
</tr>
<tr>
<td>Fresh &amp; Chopped</td>
<td>Grass Silage</td>
<td>9</td>
<td>0.5286 + -0.0007 DM (g/kg) + 0.0038 Lactic Acid (g/kgDM)</td>
<td>0.952</td>
<td>0.0455</td>
</tr>
<tr>
<td></td>
<td>Maize Silage</td>
<td>5</td>
<td>0.5036 + 0.002 DM (g/kg) + -0.0062 VFA (g/kgDM)</td>
<td>0.917</td>
<td>0.0459</td>
</tr>
<tr>
<td></td>
<td>W/crop Wheat</td>
<td>7</td>
<td>0.8906 + 0.0012 VFA (g/kgDM)</td>
<td>0.589</td>
<td>0.0261</td>
</tr>
</tbody>
</table>

NS P>0.05  *P<0.05  **P<0.01  ***P<0.001
2.5 DISCUSSION

2.5.1 EXPERIMENT 1 – MEASURING THE INDIRECT GAS PRODUCED BY THE SILAGE FERMENTATION ACIDS (SFA) OF FRESH AND DRIED GRASS SILAGE, MAIZE SILAGE AND WHOLECROP WHEAT DURING IVGPT

When investigating degradation kinetics of feeds, one of the main advantages of IVGPT is that, based on the rate of gas production in the early stages of incubation, it can provide an estimate of the quantity and rate of fermentation of QCHO. However, carbohydrate fermentation does not account for all of this initial gas production and proportion of the gas produced is due to other sources, including protein fermentation (Steingass, 1983; Cone and van Gelder, 1999), fermentation of endogenous rumen liquor carbohydrates and microbial biomass (Cone et al., 1997) and SFA gas (Deaville and Givens, 1998). To accurately describe carbohydrate degradation in feeds, it is essential that these sources of error are removed and although the interference of protein fermentation, endogenous carbohydrate and microbial turnover have all been previously considered (Steingass, 1983; Cone et al., 1997; Cone and van Gelder, 1999), very few studies have investigated effects of SFA on gas production (Deaville and Givens, 1998).

A substantial volume of gas was produced from all fresh and dried silages during the first 30 minutes of incubation in ABM, after which further gas production was within the accuracy of the technique (i.e., ±0.25ml) (Table 2.2). Because syringes were manually inoculated and read, it is possible that a proportion of the instantaneous SFA gas produced may not have been measured. However, the time required to inoculate the syringes with 30 ml of medium/inoculum and read the corresponding volume only takes 10-12 seconds, and it is assumed that the volume of gas produced in this time is so small that it is within the accuracy of the technique.

The volume and rate of indirect SFA gas production was significantly higher from fresh material of all forage types (Table 2.2), indicating that the pre-drying proposal of Deaville and Givens (1998) removes a substantial component of SFA, which is assumed to be VFA. However, a substantial volume of indirect SFA gas was also produced by dried silages, indicating that lactic acids and other non-volatile acids are not removed by drying and
consequently dried samples are still susceptible to interference by indirect SFA gas.

The level and rate of indirect SFA gas production was highest in both fresh and dried grass silage material compared to maize silage and wholecrop wheat (P>0.05) (Table 2.2), which were not significantly different (P>0.05). This indicates that the interference of indirect SFA gas production during IVGPT is highest in grass silage, which suggests that the nutritive value of grass silage may be over estimated using IVGPT.

The level of indirect SFA gas produced by fresh silages during incubation in ABM can also be used as an alternative measure of pH to predict the non-fermentative acid loading (NFAL) risk of silage in the rumen. The greatest NFAL risk is from grass silage which has the largest range of indirect SFA gas production values (4.34±1.32), followed by maize silage (2.00±0.95) and finally wholecrop wheat which has the lowest risk (1.85±0.44).

Based on the assumption that SFA are not utilised by rumen microbes (Deaville and Givens, 1998), removing the indirect gas produced by their reaction with ABM should allow more accurate prediction of the quantity of fermentable carbohydrate available, and a better appreciation of silage nutritive value as determined using IVGPT.

2.5.2 EXPERIMENT 2 – EFFECT OF SFA GAS PRODUCTION ON THE CARBOHYDRATE DEGRADATION PARAMETERS OF FRESH AND DRIED GRASS SILAGE, MAIZE SILAGE AND WHOLECROP WHEAT DURING IVGPT

Failure to account for the indirect SFA gas produced by silage during IVGPT not only resulted in the overestimation of QCHO in all fresh and dried forage material (Table 2.4; Table 2.5; Table 2.6) but also TCHO and the underestimation of SCHO. Therefore as a potential component of the QCHO fraction, the gas produced in the first 30 minutes of incubation derived from SFA has a pronounced effect on the gas production profile, directly exaggerating the quantity of QCHO and underestimating the quantity of SCHO, primarily by over prediction of the lag time.

Overestimating the quantity of fermentable carbohydrate available in silages using IVGPT could result in the formulation of asynchronous diets that not only provide insufficient total energy but are also deficient in QCHO causing quickly degradable protein (QDP) to be utilised less efficiently by rumen
microbes and, in extreme cases, risk ammonia toxicity and ketosis. The gas production model should therefore be re-written as:

\[
\text{Gas} = A(1 - \exp(-\alpha t)) + B(1 - \exp(-\beta(t-Lag))) \times \exp(5(t-Lag))/(1+\exp(5(t-Lag)))
\]

The proportion of gas produced in the first 30 minutes of IVGPT that could be attributed to indirect SFA gas was not significantly different between fresh and dried material in maize silage or wholecrop wheat (P>0.05) but was 11.8% less in fresh grass silage (60%) compared to dried grass silage (64%) (P<0.01) (Table 2.7). The proportion of indirect SFA gas was also significantly less in both fresh and dried grass silage (60% and 64%) compared to maize silage (92% and 94%) and wholecrop wheat (93% and 96%) (P<0.05), which were not significantly different (P>0.05).

Consequently after correcting for the indirect gas produced by SFA, grass silage was shown to produce significantly more gas in the first 30 minutes of IVGPT compared to maize silage and wholecrop wheat in both fresh and dried material (P<0.05). Fresh grass silage also produced 196% more gas in the first 30 minutes of IVGPT compared to the dried material, whilst there was no difference in the volume of gas produced in the first 30 minutes of IVGPT by fresh and dry maize silage or wholecrop wheat. These results indicate that not only does grass silage ferment more quickly than maize silage and wholecrop wheat, but drying under predicts the quantity of immediately fermentable carbohydrate in grass silage, presumably by altering the chemical structure of these quickly fermenting carbohydrates (Picaglia and Galleti, 1987) or by increasing the time required for hydration, attachment and colonisation by rumen microbes. This observation may also be true for maize silage and wholecrop wheat but due to the small volume of gas produced by these silages in the initial stages of incubation, any difference may be within the accuracy of the technique (±0.25 ml) and therefore immeasurable.

2.5.3 PREDICTING INDIRECT SFA GAS PRODUCTION DURING IVGPT

The results of this study show that accurate prediction of the quantity of fermentable carbohydrate in silage determined using IVGPT is only possible after correction for the indirect gas produced by SFA. Quantifying the volume of indirect SFA gas produced by individual silages by incubation in ABM would be very time consuming. An alternative approach would be to incubate the feeds overnight in ABM at 4°C and to then zero the syringes.
Again this is time consuming but also reliant on knowing the dilution ratio required in the incubation medium, which is impossible. A more economical approach is to develop equations to predict the indirect SFA gas produced during IVGPT using standard proximate analyses.

Multiple linear regression equations were developed to predict the proportion of gas produced by silages in the first 30 minutes of IVGPT that could be attributed to indirect SFA gas using DM, lactic acid and VFA as potential independent variables. Equations to predict the proportion of SFA attributable gas were successfully developed for fresh grass silage ($R^2 = 0.95$, $SE = 0.05$, $P<0.001$), dried grass silage ($R^2 = 0.71$, $SE = 0.13$, $P<0.05$), fresh wholecrop wheat ($R^2 = 0.59$, $SE = 0.03$, $P<0.05$) and dried wholecrop wheat ($R^2 = 0.59$, $SE = 0.02$, $P<0.05$) but not for fresh or dried maize silage ($P>0.05$) (Table 2.10).

Failure to predict the proportion of SFA attributed gas production for fresh and dried maize silage is because the amount of gas produced in the initial stages of IVGPT can be very low and because the accuracy of IVGPT is limited ($±0.25$ml) a relatively large number of samples were estimated to produce more gas during incubation in ABM than in rumen liquor. Samples with a SFA attributable proportions of greater than 1 were removed from the dataset and consequently the number of samples used in each multiple linear equation was very small and the accuracy of the prediction poor.

Although the level of gas production from maize silage and wholecrop wheat can be very small in the first 30 minutes of IVGPT, there is still a need for correction as maize silage in particular can produce a substantial proportion of indirect SFA gas. Further research incorporating a larger number of samples is required to improve the accuracy of these predictions, especially for maize silage and wholecrop wheat.

In contrast, the volume of indirect SFA gas produced by the fresh silages was successfully predicted because it incorporated a larger sample size, with the volume of indirect SFA gas predicted for fresh grass silage ($R^2 = 0.91$, $SE = 0.62$, $P<0.01$), fresh maize silage ($R^2 = 0.79$, $SE = 0.51$, $P<0.001$) and fresh wholecrop wheat ($R^2 = 0.61$, $SE = 0.17$, $P<0.05$) (Table 2.9)
2.6 CONCLUSION

The results of this study indicate that although pre-drying of silages significantly reduces the interference of indirect SFA gas shown during incubation of fresh material, indirect SFA gas still has a significant effect on the gas production profile. In both fresh and dried silage material, failure to account for the indirect gas attributed to SFA results in the overestimation of QCHO, lag and TCHO and the underestimation of SCHO.

The proportion of gas produced in the first 30 minutes of IVGPT that is attributed to SFA can be predicted using multiple linear equations for fresh and dried grass silage and wholecrop wheat material.

The volume of a gas produced in the first 30 minutes of IVGPT that is attributed to SFA can be predicted using multiple linear equations for fresh grass silage, fresh maize silage and fresh wholecrop wheat. This offers the potential of predicting the NFAL risk of silage and helping to formulate diets with a reduced risk of acidosis due to acidic silage.
Effect of sample preparation and particle size on the carbohydrate degradation parameters of grass silage, maize silage and wholecrop wheat using IVGPT
3.1 ABSTRACT

Forage samples are commonly dried and milled prior to routine analysis to improve handling and to produce a homogenous sample. The purpose of this investigation was to study the effect of sample processing on the carbohydrate degradation parameters of silages using IVGPT.

The particle size distribution of dried silages milled through a 1mm sieve was not dependent on forage type (P>0.05) and resulted in the majority of forage particles being less than 0.5mm and with a mean particle size of 0.18-0.25mm.

Smaller particles were fermented significantly faster during IVGPT, with a higher concentration of quickly fermentable carbohydrate (QCHO) (P<0.05) and a faster rate of fermentation of slowly fermentable carbohydrate (SCHO) (P<0.05). The totally fermentable carbohydrate (TCHO) concentration of grass silage was not affected by particle size (P>0.05), but in maize silage and wholecrop wheat the TCHO was shown to increase as particle size decreased (P<0.05) with particles less than 0.5mm also containing significantly more SCHO than larger particles (P<0.05). These results indicate that the different particle sizes of wholecrop cereals after drying and milling represent different parts of the plant and the majority of small particles are starch granules from cereal grain.

Water soluble carbohydrate was shown to account for only 80.3% of QCHO with 19.7% of QCHO attributed to insoluble carbohydrate. A proportion of soluble carbohydrate was also shown to be slowly fermented and to make up 8.5% of the SCHO fraction. Therefore it is inaccurate to describe soluble carbohydrates as QCHO and insoluble carbohydrates as SCHO.

When comparing the carbohydrate fermentation profile of fresh silages with that of dried and milled material, fresh silages contained significantly more TCHO than dried material principally related to a higher concentration of QCHO in fresh grass silage (P<0.05) and a higher concentration of SCHO in maize silage (P<0.05) and wholecrop wheat (P<0.05). There was no significant effect on the lag, but the rate of SCHO fermentation was 13.6% slower in fresh grass silage and 12.5% slower in fresh maize silage compared to dried material.
Multiple linear regression equations were developed to predict the gas production parameters of fresh forages from the gas production parameters of the same forages when dried and milled. The volume of quick gas (A) was predicted for grass silage ($R^2=90.4$, $P<0.001$), maize silage ($R^2=81.3$, $P<0.001$) and wholecrop wheat ($R^2=76.5$, $P<0.01$), as was the volume of slow gas (B) ($R^2=81.5$, $P<0.01$; $R^2=73.3$, $P<0.01$ and $R^2=84.4$, $P<0.01$ respectively), lag ($R^2=81.3$, $P<0.001$; $R^2=82.1$, $P<0.001$ and $R^2=83.8$, $P<0.01$ respectively) and C5 ($R^2=79.0$, $P<0.01$; $R^2=95.1$, $P<0.001$ and $R^2=94.2$, $P<0.001$ respectively).

The conclusion of this study is that milling of dried samples disrupts the physical characteristics of silages and by causing under-prediction of the concentration of QCHO in grass silage and the SCHO in maize silage and wholecrop wheat, ultimately causes under-prediction of the TCHO concentration of silages.
3.2 INTRODUCTION

The power of rumen models and the predicted nutrient supply of ruminant diets are dependent on accurate assessment of the chemical composition and degradation characteristics of the feeds offered. The handling of feeds prior to analysis must minimise any effect on the chemical and physical attributes of feed, but it is common in most analytical procedures, including IVGPT, for feeds to be dried at 60°C for 24 hours and milled through 1mm sieves prior to analysis. Although this creates a homogenous sample that is easily stored, analysing feeds after they have been dried and milled may be unrepresentative of how they are fed on farm where most are consumed in their fresh state, and consequently the rate and extent of digestion in the rumen may be misleading (Lowman et al., 2002).

All forms of heat drying have been shown to cause morphological changes in the chemical composition and structure of plant components (Van Soest, 1982) including a reduction in the quantity of non-structural carbohydrates (Acosta and Kothmann, 1978; Piccaglia and Galleti, 1987) and protein solubility (Pichard and Van Soest, 1977; Abdalla et al., 1988) and the denaturing of non-structural carbohydrates (Ljókel et al., 2003) and insoluble protein (Van Soest, 1982, Chamley and Viera, 1990). Heat drying of silage and fermented feeds also results in the loss of volatile alcohols, organic esters, amines and ammonia (Denium and Maassen, 1994; Snyman and Joubert, 1992) which account for a large proportion of the digestible fraction of fresh feeds (Pichard and Van Soest, 1977). Consequently the drying of these feeds results in a significant reduction in the quantity of organic matter (Acosta and Kothmann, 1978) and the digestibility of available protein and carbohydrate (Broesder et al., 1992; Bonsi et al., 1995; Nagadi et al., 1998; Valentine et al., 1999), with lower concentrations and rate of fermentation of QCHO (Cone, 1998; Nagadi et al., 1998) and faster rates of fermentation of SCH0 (Bonsi et al., 1995; Cone, 1998).

Milling dried samples also destroys the physical characteristics of a feed particularly its particle size which, along with its chemical composition, determines the rate and extent of particle breakdown and fermentation in the rumen. The critical size theory proposed by Poppi et al., (1981) suggests that feed particles can only leave the reticulo-rumen through the reticulo-omasal orifice when they are less than a critical size, which for dairy cows and steers has been suggested to be 3-4.8mm in diameter (Dixon and Milligan, 1981; Dixon and Milligan, 1985; Cordova and Mertens, 1986; Shaver et al., 1988;
Grant et al., 1990; Beauchemin et al., 1994). Because feed particles in the rumen are significantly larger than those found in dried and milled samples, milling samples through a 1mm sieve may not effectively mimic mastication and may overestimate the rate of rumen fermentation (Nagadi et al., 1998), particularly of components such as starch that are more fragile when dried.

The problem with analysing fresh material is that it is usually very difficult to grind and often results in heterogeneous samples of coarse particles which are difficult to handle, decrease the accuracy of measurement and increase the variation between samples. (Lopez et al., 1995). There is currently no standard procedure for processing fresh samples prior to analysis with fresh samples incubated whole (Nagadi, 1999), cut with scissors (Cone, 1998; Nagadi, 1999; Valentin et al., 1999) or crushed using a pestle and mortar (Nagadi, 1999) to simulate mastication. In contrast, electric blenders have been successfully employed to produce homogenous samples of frozen plant material for freeze drying (Alomar et al., 1999) and may present the ideal tool for producing homogenous sample of fresh material and allowing the study of the effect of sample processing on the degradation characteristics of samples during IVGPT.

Soluble plant components are commonly referred to as being smaller than <20-25μm (Dawson and Steen, 2000) and although shown to be fermented at a faster rate than the majority of insoluble components, there is evidence to suggest that not all soluble components are quickly fermented in the rumen (Stefanon et al., 1996). Because the liquid passage rate of soluble components out of the rumen is significantly faster than that of larger insoluble components, a proportion of plant solubles may pass out of the rumen undegraded. This has a pronounced effect on the application of degradation parameters estimated using IVGPT because this technique incorporates no dynamic effects of rumen flow. If a proportion of SCHO estimated using IVGPT is derived from soluble carbohydrates, depending on the lag time and liquid passage rate, a proportion of this SCHO may be unavailable in the rumen and absorbed directly in the small intestine.

The aims of this study were to (1) measure the particle size distribution of dried and milled silages and to estimate the degradation parameters of each particle size using IVGPT, (2) estimate the proportion of soluble carbohydrate that is quickly fermented during IVGPT, and (3) compare the estimated degradation parameters of fresh and dried silages during IVGPT and to develop equations to predict the degradation kinetics of fresh silage using dried material.
3.3 MATERIAL AND METHODS

3.3.1 EXPERIMENTAL TREATMENTS

EXPERIMENT 1

This experiment was designed to measure the particle size distribution of dried grass silage, maize silage and wholecrop wheat milled through a 1mm sieve and to assess the carbohydrate fermentation parameters of each particle size using IVGPT

EXPERIMENT 2

This experiment was designed to determine what proportion of water soluble silage carbohydrates are quickly fermented by incubating washed and unwashed silage samples in IVGPT.

EXPERIMENT 3

This experiment was designed to compare the carbohydrate fermentation profile of fresh and dried silages during IVGPT and to develop equations to predict the carbohydrate fermentation parameters of fresh silages using dried material.

3.3.2 PROXIMATE ANALYSIS

Samples of 11 grass silages made from predominantly perennial ryegrass swards, 19 maize silages, and 11 wholecrop wheat samples, all cut in 2003, were obtained from commercial farms throughout the United Kingdom (UK) by Bioparametrics Ltd., an Edinburgh based analytical company interested in developing IVGPT for commercial applications.

The DM content of each forage sample was measured by drying approximately 100g of fresh material in a 60°C oven for 24 hours. The dried samples were then milled through a 1mm sieve using a Retsch® mill, analysed for NDF (Van Soest et al., 1991) and ADF using ANKOM F57® fibre bags and crude protein (CP) using the standard Kjedahl technique. See Appendix A.1 for details of the methodology used. The lactic acid (g/kgDM), total volatile fatty acid (VFA) (g/kgDM) content and pH of each of the silages
was estimated by NIRS (Foss NIRSystem 6500) using calibrations developed by the Forage Analysis Assurance FAA (FAA) group.

To simulate mastication, fresh forage samples were frozen overnight and then approximately 15g of the frozen material was chopped in a Moulinex Ovatio 3 Duo blender at 1450rpm for 15 seconds.

3.3.3 MEASUREMENT OF THE PARTICLE SIZE DISTRIBUTION OF DRIED AND MILLED SILAGE SAMPLES

The particle size distribution of dried and milled silage was measured by dry sieving approximately 15g of material using a Retsch Sieve Shaker AS200 through a test sieve set of decreasing aperture size (1mm, 0.85mm, 0.50mm, 0.25mm).

The mean particle size was calculated assuming that the mean particle size retained on each test sieve was equal to that of the sieves aperture size. For the smallest particles that were not retained on any sieve it was assumed that the mean particle size was half the aperture size of the final sieve:

\[
\text{Mean particle size (mm)} = ((1.00\times \text{retained sample(g)}) + (0.85\times \text{retained sample(g)}) + (0.50\times \text{retained sample(g)}) + (0.25\times \text{retained sample(g)}) + ((0.25/2)\times \text{retained sample(g)}))/100
\]

3.3.4 MEASUREMENT OF THE SOLUBLE DM CONTENT OF DRIED AND MILLED SILAGE SAMPLES

Approximately 500mg of each silage sample was weighed in duplicate into ANKOM F57© fibre bags and sealed using a heat sealer. Allowing approximately 50ml for each bag, the bags were washed with warm distilled water for 15 minutes using a magnetic stirrer. The distilled water was poured off and the bags washed at least twice more in warm distilled water or until there was no further colour change in the water. The water was poured off and, without being squeezed, the bags were placed in a 60°C oven for 24 hours, cooled in a desiccator, reweighed and the DM loss calculated.
3.3.5 ESTIMATING THE RUMEN FERMENTATION PROFILE USING IVGPT

After simulated mastication, the fresh samples were allowed to defrost at room temperature for twenty minutes. The equivalent of 220 mg DM of dry or fresh silage were weighed in duplicate into 100 ml glass syringes and placed in the fridge until the morning of incubation. Three additional syringes were also created and placed in the fridge but contained no samples.

On the morning of inoculation and prior to collecting the rumen liquor, the syringes containing the samples, along with ‘blanks’ containing no substrate, were placed in a 39°C water bath.

Rumen liquor was collected from sheep kept outside during summer and grazing predominantly perennial ryegrass with no access to supplemental feeds. One sheep was used for each gas production run. On the morning of inoculation, individual sheep were collected from the pasture at approximately 09:30, slaughtered and the rumen and reticulum removed and placed in a thermos box to keep warm whilst travelling back to the laboratory. At the laboratory the rumen contents were strained through two layers of 250 μm netting and three layers of muslin, and then stored in a thermos flask gassed with CO₂.

To ensure an adequate microbial population is incubated with each sample and to enable comparison of samples in different gas production runs, microbial activity of the rumen liquor was assessed by measuring its absorbance at 600 nm (Nagadi et al., 2000). The dilution ratio with anaerobic medium was then calculated to ensure that the inoculum had an absorbance at 600 nm of 0.111 - 0.134, thereby ensuring that microbial activity among the IVGPT runs was similar.

The appropriate volume of ABM (Menke and Steingass, 1988) was prepared, placed in a 39°C water bath and saturated with CO₂. When the medium reached 39°C, rumen liquor was added and the inoculum was gassed with CO₂ for a further 5 minutes. Using an automatic dispensing pump, 30 ml of inoculum was added to each fermentation syringe before it was returned to the water bath. Measurement of in vitro gas production were performed as described by Jessop and Herrero (1996), with cumulative gas production in each syringe read every half hour for the first 3 hours, then hourly until 6 hours and thereafter at 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 56, 64, and 72, 80, 88 and 96h. Syringes were manually shaken at each reading.
3.3.6 INTERPRETATION OF GAS PRODUCTION DATA

The fermentation profile of each silage and/or particle size was determined by applying the analytical procedure described in Appendix A1.4.

This initially describe the gas profile of each silage in terms of the volume (A) and rate of gas (Cₐ) produced in the early stages incubation and then after a lag, the volume (B) and rate of gas (Cₛ) produced in the later stages of incubation. The gas profile is then transformed to reflect the quantity and rate of QCHO and SCHO fermented.

3.3.7 ESTIMATING THE DEGRADATION PARAMETERS OF SOLUBLE FORAGE CARBOHYDRATE

Soluble CHO = (((unwashed A (ml)-(washed A (ml)*(soluble DM (g/kgDM)/1000))/ unwashed A (ml))*100

(% QCHO fraction)

Insoluble CHO= 100 - (((unwashed A (ml)-(washed A (ml)*(soluble DM

(% QCHO fraction) (g/kgDM)/1000))/unwashed A (ml))*100

Soluble CHO = (((unwashed B (ml)-(washed B (ml)*(soluble DM (g/kgDM)/1000))/ unwashed B (ml))*100

(% SCHO fraction)

Insoluble CHO= 100-(((unwashed B (ml)-(washed B (ml)*(soluble DM

(% SCHO fraction) (g/kgDM)/1000))/unwashed B (ml))*100

3.3.8 STATISTICAL ANALYSIS

Using the MINITAB statistical package (2000), paired T-tests were used to compare the cumulative gas production and degradation parameters of the different particle sizes, the washed and unwashed silages and the dried and fresh silages during IVGPT. Analysis of variance (ANOVA) using a general linear model (GLM) was used to compare the difference between different silage types. Multiple linear regressions were used to predict the gas production parameters of fresh silages using proximate results and the gas production parameters of dried silages as independent variables. The independent variables used for each forage type was selected using the ‘best subset’ function within the statistical software (Minitab, 2000). Outliers were removed using the ‘unusual observation function’ within the Minitab (2000), which removes observations with a standardized residual greater than 2.
3.4 RESULTS

3.4.1 EXPERIMENT 1 – EFFECT OF PARTICLE SIZE ON THE DEGRADATION PARAMETERS OF DRIED AND MILLED FORAGES ESTIMATED USING IVGPT

3.4.1.1 THE PARTICLE SIZE DISTRIBUTION OF DRIED SILAGES MILLED THROUGH A 1 MM SIEVE

The proximate analysis and particle size distribution of the dried silage samples studied are represented in Table 3.1.

With the exception of particles greater than 1mm, where maize silage had a significantly higher proportion (P>0.05) compared to grass silage and wholecrop wheat, there was no significant difference between the particle size distribution of the different silages. The majority of particles in all the silages were less than 0.5 mm in diameter with a mean particle size of 0.18-0.21mm.

Table 3.1 Proximate Analyses and particle size distribution of dry grass silage, maize silage and wholecrop wheat milled through a 1mm sieve. Standard deviation values are shown in brackets. For each forage type, means with different superscripts within the same row are significantly different (P<0.05).

<table>
<thead>
<tr>
<th>Type</th>
<th>Grass silage</th>
<th>Maize silage</th>
<th>Wholecrop Wheat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>DM (g/kg)</td>
<td>312.2 (117.2)</td>
<td>310.8 (42.6)</td>
<td>404.6 (33.2)</td>
</tr>
<tr>
<td>ADF (g/kgDM)</td>
<td>345.6 (27.6)</td>
<td>243.6 (21.7)</td>
<td>269.5 (40.0)</td>
</tr>
<tr>
<td>NDF (g/kgDM)</td>
<td>475.3 (88.4)</td>
<td>389.1 (52.9)</td>
<td>382.2 (45.3)</td>
</tr>
<tr>
<td>&gt;1.0mm (%)</td>
<td>0.13 (0.045)</td>
<td>0.45 (0.092)</td>
<td>0.21 (0.053)</td>
</tr>
<tr>
<td>0.85 to 1.0mm (%)</td>
<td>0.58 (0.416)</td>
<td>1.28 (0.50)</td>
<td>1.21 (0.379)</td>
</tr>
<tr>
<td>0.5 to 0.85mm (%)</td>
<td>5.66 (1.109)</td>
<td>5.95 (3.62)</td>
<td>8.41 (2.48)</td>
</tr>
<tr>
<td>0.25 to 0.5mm (%)</td>
<td>27.65 (0.512)</td>
<td>44.69 (1.23)</td>
<td>34.21 (8.38)</td>
</tr>
<tr>
<td>&lt;0.25mm (%)</td>
<td>65.07 (1.00)</td>
<td>42.56 (5.72)</td>
<td>53.10 (4.08)</td>
</tr>
<tr>
<td>Mean (mm)</td>
<td>0.18 (0.007)</td>
<td>0.21 (0.019)</td>
<td>0.21 (0.0003)</td>
</tr>
</tbody>
</table>

NS: P>0.05  *P<0.05  **P<0.01  ***P<0.001
3.4.1.2 THE EFFECT OF PARTICLE SIZE ON THE CARBOHYDRATE FERMENTATION PARAMETERS OF DRIED AND MILLED SILAGE

The estimated carbohydrate fermentation parameters of the different particles sizes of dried and milled grass silage, maize silage and wholecrop wheat are presented in Table 3.2 and illustrated in Fig 3.1, Fig. 3.2 and Fig 3.3.

All forage particles less than 0.25mm produced significantly more gas in the initial stages of incubation and were assumed to be more quickly fermented than larger particles (>0.25mm)

The level of gas produced in the later stages of incubation that is related to slowly fermented material was significantly higher in maize silage and wholecrop wheat particles less than 0.5mm (P<0.05) and in grass silage particle greater than 0.25mm (P<0.05). All forage particles less than 0.5mm also had a shorter lag (P<0.05) and as the particle size decreased the rate of gas produced by slowly fermentable plant components increased (P<0.05)

There was no significant difference in the total volume of gas produced by the different grass silage particle sizes (P<0.05), but total gas production increased as the particle size of maize silage decreased (P<0.05) and was significantly higher in wholecrop wheat particles less than 0.25mm (P<0.05).
Table 3.2 Estimated gas production degradation parameters of different particle sizes of dried and milled grass silage (n=2), maize silage (n=2) and wholecrop Wheat (n=2) incubated in duplicate in ovine ruminal fluid. The cumulative gas production curves were fitted to the equation: $\text{Gas} = A(1-\exp(-c_{\text{t}})+B(1-\exp(-c(t-Lag)))*\exp(5(t-Lag))/(1+\exp(5(t-Lag)))$ modified from Jessop and Herrero (1996). [see text for interpretation of parameters]. Standard deviation values are shown in brackets. For each particle size, means with different superscripts within the same row are significantly different (P<0.05).

<table>
<thead>
<tr>
<th>Type</th>
<th>Parameter</th>
<th>Whole</th>
<th>&gt;0.85</th>
<th>0.5-0.85</th>
<th>0.25-0.5</th>
<th>&lt;0.25</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DM Proportion (%)</td>
<td>100</td>
<td>0.56 (0.42)</td>
<td>5.71 (1.12)</td>
<td>27.91 (0.53)</td>
<td>65.67 (1.04)</td>
</tr>
<tr>
<td>Grass Silage</td>
<td>A (ml)</td>
<td>15.25 (2.67)</td>
<td>13.31 (6.22)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.47 (2.61)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.76 (1.78)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>19.60 (2.80)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Ca (h&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.296 (0.005)</td>
<td>0.250 (0.071)</td>
<td>0.25 (0.071)</td>
<td>0.242 (0.042)</td>
<td>0.205 (0.007)</td>
</tr>
<tr>
<td></td>
<td>B (ml)</td>
<td>36.73 (5.10)</td>
<td>44.09 (14.10)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.15 (9.03)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.06 (6.19)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>33.01 (5.63)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Cb (h&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.075 (0.004)</td>
<td>0.071 (0.013)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.059 (0.005)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.072 (0.005)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.083 (0.001)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Lag (h)</td>
<td>7.32 (0.19)</td>
<td>8.96 (0.16)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.36 (1.61)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.87 (1.19)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.99 (0.038)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Total Gas (ml)</td>
<td>51.98 (4.54)</td>
<td>57.40 (7.87)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.63 (6.32)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>54.82 (4.42)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>52.60 (2.83)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maize Silage</td>
<td>DM Proportion (%)</td>
<td>100</td>
<td>1.35 (0.53)</td>
<td>6.28 (3.83)</td>
<td>47.08 (1.43)</td>
<td>44.82 (5.90)</td>
</tr>
<tr>
<td></td>
<td>A (ml)</td>
<td>17.62 (3.81)</td>
<td>14.28 (2.56)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.45 (1.35)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.04 (4.79)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.30 (3.87)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Ca (h&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.223 (0.014)</td>
<td>0.221 (0.030)</td>
<td>0.229 (0.042)</td>
<td>0.3 (0.0)</td>
<td>0.256 (0.005)</td>
</tr>
<tr>
<td></td>
<td>B (ml)</td>
<td>48.46 (1.71)</td>
<td>48.47 (0.38)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.93 (0.01)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.77 (0.09)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>56.08 (0.88)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Cb (h&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.068 (0.002)</td>
<td>0.051 (0.008)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.053 (0.03)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.062 (0.002)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.099 (0.003)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Lag (h)</td>
<td>2.64 (0.15)</td>
<td>9.36 (0.07)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.17 (0.22)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.78 (0.42)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.79 (0.19)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Total Gas (ml)</td>
<td>66.08 (5.52)</td>
<td>62.84 (2.95)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.37 (1.35)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>64.80 (4.70)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72.38 (2.99)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wholecrop Wheat</td>
<td>DM Proportion (%)</td>
<td>100</td>
<td>1.24 (0.41)</td>
<td>8.68 (2.67)</td>
<td>35.16 (8.13)</td>
<td>54.70 (4.99)</td>
</tr>
<tr>
<td></td>
<td>A (ml)</td>
<td>21.92 (0.80)</td>
<td>9.86 (6.40)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.90 (2.66)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.42 (7.25)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.72 (4.09)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Ca (h&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.215 (0.021)</td>
<td>0.30 (0.0)</td>
<td>0.25 (0.07)</td>
<td>0.30 (0.0)</td>
<td>0.228 (0.016)</td>
</tr>
<tr>
<td></td>
<td>B (ml)</td>
<td>37.66 (11.31)</td>
<td>37.60 (3.42)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.21 (2.90)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>45.67 (6.55)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>46.94 (5.40)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Cb (h&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0.0528 (0.014)</td>
<td>0.0392 (0.0112)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0399 (0.007)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0443 (0.011)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.0691 (0.011)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Lag (h)</td>
<td>4.41 (2.96)</td>
<td>5.97 (1.79)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.20 (1.07)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.01 (0.49)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.37 (0.11)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Total Gas (ml)</td>
<td>59.67 (10.39)</td>
<td>47.46 (9.82)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52.11 (9.76)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>57.08 (13.91)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>64.68 (9.49)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
**Fig 3.1** Cumulative gas production of the different particle sizes of dried grass silage milled through a 1mm sieve and 220mgDM incubated in duplicate (n=2) in ovine rumen liquor. Particle sizes are: whole (cross), >0.85mm (white diamond), 0.5-0.85mm (black circle), 0.25-0.5mm (black square), <0.25mm (white square). Cumulative gas production curves were fitted to the equation: \( \text{Gas} = A(1-\exp(-c_d)) + B(1-\exp(-c(t-Lag))) \exp(5(t-Lag))/(1+\exp(5(t-Lag))) \) modified from Jessop and Herrero (1996) [see text for interpretation of parameters].

**Fig 3.2** Cumulative gas production of the different particle sizes of dried maize silage milled through a 1mm sieve and 220mgDM incubated in duplicate (n=2) in ovine rumen liquor. Particle sizes are: whole (cross), >0.85mm (white diamond), 0.5-0.85mm (black circle), 0.25-0.5mm (black square), <0.25mm (white square). Cumulative gas production curves were fitted to the equation: \( \text{Gas} = A(1-\exp(-c_d)) + B(1-\exp(-c(t-Lag))) \exp(5(t-Lag))/(1+\exp(5(t-Lag))) \) modified from Jessop and Herrero (1996) [see text for interpretation of parameters].
3.4.2 EXPERIMENT 2 – ESTIMATING THE CONTRIBUTION OF WATER SOLUBLE CARBOHYDRATES TO THE QCHO FRACTION OF SILAGE DURING IVGPT

3.4.2.1 THE PROPORTION OF WATER SOLUBLE DM IN DRIED AND MILLED SILAGE

The concentration of water soluble DM was not significantly different between dried and milled samples of grass silage, maize silage and wholecrop wheat (Table 3.3)
Table 3.3 Mean particle size and solubility of dry grass silage, maize silage and wholecrop wheat milled through a 1mm sieve. Standard deviation values are shown in brackets. For each forage type, means with different superscripts within the same row are significantly different (P<0.05).

<table>
<thead>
<tr>
<th>Type</th>
<th>Grass silage</th>
<th>Maize silage</th>
<th>Wholecrop Wheat</th>
<th>Type Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>NS</td>
</tr>
<tr>
<td>Soluble DM (g/kgDM)</td>
<td>357.2 (89.7)</td>
<td>225.7 (5.20)</td>
<td>308.0 (165.0)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS P>0.05  *P<0.05  **P<0.01  ***P<0.001

3.4.2.2 THE CONTRIBUTION OF WATER SOLUBLE CARBOHYDRATE TO THE QCHO FRACTION OF SILAGE DURING IVGPT

The gas production parameters of the washed and unwashed silage samples during IVGPT are presented in Table 3.4 and illustrated in Fig.3.4. The contribution of soluble carbohydrate to the estimated QCHO and SCHF silage fractions are presented in Table 3.5.

There was no significant difference in the lag, volume (B) or rate (Cₜ) of gas produced by slowly fermentable carbohydrates in washed and unwashed silage samples (P>0.05). However, significantly more gas was produced by the unwashed silage samples in the initial stages of incubation (A) (P<0.001) and in total (A plus B) (P<0.01).

These results indicate that regardless of forage type (P>0.05), soluble carbohydrate accounts for only 79.6% of QCHO (Table 3.4) with 19.7% attributed to quickly fermenting insoluble carbohydrates (Table 3.5). In addition, only 80.3% of soluble silage carbohydrates are quickly fermenting with up to 23% being slowly fermentable and possibly contributing to the bypass carbohydrate fraction.
Table 3.4 Estimated gas production degradation parameters of 220mgDM of dry and milled grass silage, maize silage and wholecrop wheat incubated in ovine rumen liquor whole or after being washed with distilled water. Samples were incubated in duplicate (n=2). Cumulative gas production curves were fitted to the equation: $\text{Gas} = A(1-\exp(-ctl)) + B(1-\exp(-ct(L-lag)))^{*}\exp(5(t-Lag)) / (1 + \exp(5(t-Lag)))$ modified from Jessop and Herrero (1996) [see text for interpretation of parameters]. Standard deviation values are shown in brackets.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Unwashed</th>
<th>Washed</th>
<th>Sig.</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>6</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (ml)</td>
<td>18.26 (3.69)</td>
<td>3.73 (3.77)</td>
<td>***</td>
<td>79.6</td>
</tr>
<tr>
<td>Ca (/h)</td>
<td>0.245 (0.0417)</td>
<td>0.235 (0.0473)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B (ml)</td>
<td>40.95 (8.40)</td>
<td>38.57 (8.09)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Cb (/h)</td>
<td>0.065 (0.0123)</td>
<td>0.064 (0.00781)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Lag</td>
<td>4.79 (2.50)</td>
<td>3.41 (3.24)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Total Gas (ml)</td>
<td>59.22 (8.49)</td>
<td>42.30 (8.81)</td>
<td>**</td>
<td>28.6</td>
</tr>
</tbody>
</table>

NS P>0.05  *P<0.05  **P<0.01  ***P<0.001

Fig 3.4 Mean cumulative gas production of washed (white square) and unwashed (black triangle) dried and milled grass silage, maize silage and wholecrop wheat incubated in duplicate (n=2) in ovine rumen liquor. Cumulative gas production curves were fitted to the equation: $\text{Gas} = A(1-\exp(-ctl)) + B(1-\exp(-ct(L-lag)))^{*}\exp(5(t-Lag)) / (1 + \exp(5(t-Lag)))$ modified from Jessop and Herrero (1996) [see text for interpretation of parameters].
Table 3.5 The contribution of soluble and insoluble silage carbohydrate to the QCHO and SCHO fractions of dried and milled grass silage, maize silage and wholecrop wheat incubated in ovine rumen liquor using IVGPT. The samples were incubated in duplicate (n=2) unwashed or after being washed in distilled water. Standard deviation values are shown in brackets.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
<th>Type Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble CHO (% QCHO fraction)</td>
<td>80.30 (18.16)</td>
<td>60.22</td>
<td>100</td>
<td>NS</td>
</tr>
<tr>
<td>Insoluble CHO (% QCHO fraction)</td>
<td>19.70 (18.16)</td>
<td>0</td>
<td>39.78</td>
<td>NS</td>
</tr>
<tr>
<td>Soluble CHO (% SCHO fraction)</td>
<td>8.52 (8.47)</td>
<td>0</td>
<td>23.01</td>
<td>NS</td>
</tr>
<tr>
<td>Insoluble CHO (% SCHO fraction)</td>
<td>91.48 (8.47)</td>
<td>76.99</td>
<td>100</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS P>0.05  *P<0.05  **P<0.01  ***P<0.001

3.4.3 EXPERIMENT 3 – COMPARISON OF THE CARBOHYDRATE FERMENTATION PARAMETERS OF FRESH AND DRIED SILAGES ESTIMATED USING IVGPT

3.4.3.1 PROXIMATE ANALYSIS

The proximate analyses results for the forage samples studied are presented in Table 3.6.

Table 3.6 Proximate analyses of grass silage, maize silage and wholecrop wheat. Standard deviation values are shown in brackets.

<table>
<thead>
<tr>
<th>Type</th>
<th>Grass silage</th>
<th>Maize silage</th>
<th>Wholecrop Wheat</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of samples</td>
<td>11</td>
<td>19</td>
</tr>
<tr>
<td>DM (g/kg)</td>
<td></td>
<td>298.0 (87.2)</td>
<td>280.1 (48.3)</td>
</tr>
<tr>
<td>ADF (g/kgDM)</td>
<td></td>
<td>311.2 (43.3)</td>
<td>266.7 (24.41)</td>
</tr>
<tr>
<td>NDF (g/kgDM)</td>
<td></td>
<td>468.4 (61.2)</td>
<td>407.9 (38.2)</td>
</tr>
<tr>
<td>CP (g/kgDM)</td>
<td></td>
<td>122.4 (22.83)</td>
<td>72.3 (9.86)</td>
</tr>
<tr>
<td>Lactic Acid (g/kgDM)</td>
<td></td>
<td>84.23 (28.20)</td>
<td>22.83 (17.67)</td>
</tr>
<tr>
<td>VFAcid (g/kgDM)</td>
<td></td>
<td>34.30 (34.1)</td>
<td>26.96 (16.68)</td>
</tr>
</tbody>
</table>
3.4.3.2 CARBOHYDRATE FERMENTATION PARAMETERS OF FRESH AND DRIED SILAGES DURING IVGPT

The carbohydrate fermentation parameters of fresh and dried grass silage, maize silage and wholecrop wheat are presented in Table 3.7 and Table 3.8 and the cumulative gas production profiles illustrated in Fig 3.6, Fig 3.7 and Fig 3.8.

There was no significant difference in the concentration of QCHO between fresh and dried maize silage and wholecrop wheat (P>0.05), but QCHO was 36.9% higher in fresh grass silage compared to the dried and milled material (P<0.001) (Table 3.7). Grass silage also contained significantly more QCHO than maize silage and wholecrop wheat in both the fresh and dried material (P>0.05)

There was no significant difference in the concentration of SCHO between fresh and dried grass silage (P>0.05), but SCHO was 6.7% higher (P<0.01) in fresh maize silage and 15.7% higher (P<0.01) in fresh wholecrop wheat compared to the dried and milled material. The concentration of SCHO was shown to be highest in maize silage, followed by wholecrop wheat and finally grass silage which had the lowest concentration of SCHO when both fresh and dried (P<0.05).

There was no significant effect of forage type or form on the lag (P>0.05) but the rate of fermentation of SCHO was 13.6% (P<0.01) lower in fresh grass silage and 12.5% lower (P<0.001) in fresh maize silage compared to the dried material. The rate of fermentation of SCHO was not significantly different between fresh and dried wholecrop wheat (P>0.05) which were also shown to ferment at a rate significantly slower that that of both fresh and dried grass silage and maize silage (P<0.05).
Table 3.7 Comparison of the estimated degradation parameters of fresh and dried grass silage ($n=14$), maize silage ($n=19$) and wholecrop wheat ($n=11$) incubated in ovine rumen liquor and corrected for SFA gas production. Each sample was incubated in duplicate ($n=2$). Dried samples were dried at 60°C for 24h and milled through a 1mm screen. Fresh samples were chopped in a Moulinex Ovatio 3 Duo blender prior to incubation. Standard deviation values are shown in brackets.

<table>
<thead>
<tr>
<th>Type</th>
<th>Parameters</th>
<th>Dry &amp; Milled</th>
<th>Fresh &amp; Chopped</th>
<th>Sig.</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass Silage</td>
<td>QCHO (g/kgDM)</td>
<td>204.2 (67.7)</td>
<td>279.7 (66.7)</td>
<td>***</td>
<td>+36.9</td>
</tr>
<tr>
<td></td>
<td>Ca (/h)</td>
<td>0.200 (0.000)</td>
<td>0.202 (0.004)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SCHO (g/kgDM)</td>
<td>338.4 (57.0)</td>
<td>377 (71.7)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cb (/h)</td>
<td>0.088 (0.025)</td>
<td>0.076 (0.015)</td>
<td>**</td>
<td>-13.6</td>
</tr>
<tr>
<td></td>
<td>Lag (h)</td>
<td>5.64 (1.34)</td>
<td>5.18 (1.64)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCHO (g/kgDM)</td>
<td>501.4 (12.5)</td>
<td>601.3 (17.6)</td>
<td>***</td>
<td>+19.9</td>
</tr>
<tr>
<td>Maize Silage</td>
<td>QCHO (g/kgDM)</td>
<td>110.9 (44.3)</td>
<td>120.8 (57.1)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ca (/h)</td>
<td>0.201 (0.002)</td>
<td>0.200 (0.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SCHO (g/kgDM)</td>
<td>571.2 (49.8)</td>
<td>609.5 (71.8)</td>
<td>**</td>
<td>+6.7</td>
</tr>
<tr>
<td></td>
<td>Cb (/h)</td>
<td>0.082 (0.013)</td>
<td>0.072 (0.010)</td>
<td>***</td>
<td>-12.5</td>
</tr>
<tr>
<td></td>
<td>Lag (h)</td>
<td>5.12 (1.05)</td>
<td>5.05 (0.95)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCHO (g/kgDM)</td>
<td>659.8 (8.6)</td>
<td>705.9 (11.4)</td>
<td>**</td>
<td>+6.9</td>
</tr>
<tr>
<td>Wholecrop Wheat</td>
<td>QCHO(g/kgDM)</td>
<td>91.8 (36.6)</td>
<td>101.0 (45.8)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ca (/h)</td>
<td>0.200 (0.0)</td>
<td>0.200 (0.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SCHO (g/kgDM)</td>
<td>437.2 (16.1)</td>
<td>506 (38.8)</td>
<td>**</td>
<td>+15.7</td>
</tr>
<tr>
<td></td>
<td>Cb (/h)</td>
<td>0.0622 (0.006)</td>
<td>0.0573 (0.005)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lag (h)</td>
<td>5.34 (0.59)</td>
<td>5.37 (0.59)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCHO (g/kgDM)</td>
<td>510.5 (37.9)</td>
<td>586.6 (43.8)</td>
<td>**</td>
<td>+14.9</td>
</tr>
</tbody>
</table>

NS P>0.05  *P<0.05  **P<0.01  ***P<0.001
Fig 3.5 Mean cumulative gas production of fresh and chopped (white square) and dried and milled (black triangle) grass silage (n=14) incubated in duplicate (n=2) in ovine rumen liquor and corrected for SFA gas production. Cumulative gas production curves were fitted to the equation: \[ \text{Gas} = A(1-\exp(-ct)) + B(1-\exp(-ct-Lag))\exp(5(t-Lag))/(1+\exp(5(t-Lag))) \] modified from Jessop and Herrero (1996) [see text for interpretation of parameters].

Fig 3.6 Mean cumulative gas production of fresh and chopped (white square) and dried and milled (black triangle) maize silage (n=19) incubated in duplicate (n=2) in ovine rumen liquor and corrected for SFA gas production. Cumulative gas production curves were fitted to the equation: \[ \text{Gas} = A(1-\exp(-ct)) + B(1-\exp(-ct-Lag))\exp(5(t-Lag))/(1+\exp(5(t-Lag))) \] modified from Jessop and Herrero (1996) [see text for interpretation of parameters].
**Fig 3.7** Mean cumulative gas production of fresh and chopped (white square) and dried and milled (black triangle) wholecrop wheat ($n=11$) incubated in duplicate ($n=2$) in ovine rumen liquor and corrected for SFA gas production. Cumulative gas production curves were fitted to the equation: 

$$\text{Gas} = A(1-\exp(-ct)) + B(1-\exp(-c(t-Lag)))\exp(5(t-Lag))/(1+\exp(5(t-Lag)))$$

modified from Jessop and Herrero (1996) [see text for interpretation of parameters].

**Table 3.8** Comparison of the estimated degradation parameters of fresh and dried grass silage ($n=14$), maize silage ($n=19$) and wholecrop wheat ($n=11$) incubated in ovine rumen liquor and corrected for SFA gas production. Each sample was incubated in duplicate ($n=2$). Standard deviation values are shown in brackets. For each forage type, means with different superscripts within the same row are significantly different ($P<0.05$).

<table>
<thead>
<tr>
<th>Type</th>
<th>Parameters</th>
<th>Grass silage</th>
<th>Maize silage</th>
<th>Wholecrop wheat</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QCHO (g/kgDM)</td>
<td>204.2 (67.7)$^a$</td>
<td>110.9 (44.3)$^b$</td>
<td>91.8 (36.6)$^b$</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Ca (h)</td>
<td>0.200 (0.00)</td>
<td>0.201 (0.002)</td>
<td>0.200 (0.000)</td>
<td></td>
</tr>
<tr>
<td>Dry &amp; Milled</td>
<td>SCHO (g/kgDM)</td>
<td>338.4 (57.0)$^a$</td>
<td>571.2 (49.8)$^b$</td>
<td>437.2 (0.006)$^c$</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Cb (h)</td>
<td>0.088 (0.025)$^a$</td>
<td>0.082 (0.013)$^a$</td>
<td>0.062 (0.006)$^c$</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Lag (h)</td>
<td>5.64 (1.34)</td>
<td>5.12 (1.05)</td>
<td>5.34 (0.59)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>TCHO (g/kgDM)</td>
<td>501.4 (12.5)$^a$</td>
<td>659.8 (8.6)$^b$</td>
<td>510.5 (37.9)$^a$</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>QCHO (g/kgDM)</td>
<td>279.7 (66.7)$^a$</td>
<td>120.8 (57.1)$^b$</td>
<td>101.0 (45.8)$^b$</td>
<td>***</td>
</tr>
<tr>
<td>Fresh &amp; Chopped</td>
<td>Ca (h)</td>
<td>0.202 (0.004)</td>
<td>0.200 (0.000)</td>
<td>0.200 (0.000)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SCHO (g/kgDM)</td>
<td>377.0 (71.7)$^a$</td>
<td>609.5 (71.8)$^b$</td>
<td>506 (38.8)$^c$</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Cb (h)</td>
<td>0.076 (0.025)$^a$</td>
<td>0.072 (0.010)$^a$</td>
<td>0.057 (0.005)$^b$</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Lag (h)</td>
<td>5.18 (1.64)</td>
<td>5.05 (0.95)</td>
<td>5.37 (0.59)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>TCHO (g/kgDM)</td>
<td>601.3 (17.6)$^a$</td>
<td>705.9 (11.4)$^b$</td>
<td>586.6 (43.8)$^a$</td>
<td>***</td>
</tr>
</tbody>
</table>

$^a$ $P>0.05$  $^b$ $P<0.05$  $^c$ $P<0.01$  $^d$ $P<0.001$
Fig 3.8 Mean cumulative gas production of fresh and chopped grass silage (n=14) (black circle), maize silage (n=19) (white square) and wholecrop wheat (n=11) (white diamond) incubated in duplicate (n=2) in ovine rumen liquor and corrected for SFA gas production. Cumulative gas production curves were fitted to the equation: \( \text{Gas} = A(1-\exp(-c.t)) + B(1-\exp(-\alpha(t-Lag))) \times \exp(5(t-Lag))/(1+\exp(5(t-Lag))) \) modified from Jessop and Herrero (1996) [see text for interpretation of parameters].

3.4.3.3 PREDICTING THE CARBOHYDRATE FERMENTATION PARAMETERS OF FRESH SILAGES USING DRIED MATERIAL

Assuming that the rate of fermentation of QCHO is 0.2 h\(^{-1}\) (see Appendix A.2 for details), multiple regression equations were successfully developed to predict the gas production parameters of fresh silage when using dried material during IVGPT. The volume of gas produced by QCHO (A) in fresh silage can be predicted for grass silage (\( R^2=90.4, P<0.001 \)), maize silage (\( R^2=0.813, P<0.001 \)) and wholecrop (\( R^2=0.765, P<0.01 \)) as well as the volume of gas produced by SCHO (B) (\( R^2=81.5, P<0.01; R^2=73.3, P<0.01; R^2=84.4, P<0.01 \)), the respective lag (\( R^2=0.813, P<0.001; R^2=0.821, P<0.001; R^2=0.838, P<0.01 \)) and rate of SCHO fermentation (\( C_b \)) (\( R^2=0.79, P<0.01; R^2=0.951, P<0.001; R^2=0.942, P<0.001 \)) (Table 3.9).
Table 3.9 Multiple linear regression equations to predict the gas production parameters of fresh grass silage, maize silage and wholecrop wheat from standard analysis values and the estimated gas production parameters of 220mgDM of dried grass silage (n=11), maize silage (n=14) and wholecrop wheat (n=11) when incubated in ovine rumen liquor. SEE is the standard error (+/-) of each estimated parameter.

<table>
<thead>
<tr>
<th>Type</th>
<th>Equation</th>
<th>Mean</th>
<th>$R^2$</th>
<th>SEE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (ml)</td>
<td>15.8 + 0.059 a (ml) + 0.0523 DM (g/kg) - 0.013 NDF (g/kgDM)</td>
<td>24.9</td>
<td>0.904</td>
<td>2.17</td>
<td>***</td>
</tr>
<tr>
<td>Ca (l/h)</td>
<td>0.2</td>
<td>0.202</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B (ml)</td>
<td>-95.1 + 0.0941 DM (g/kg) + 0.0866 NDF (g/kgDM) + 0.241 Lactic acid (g/kgDM) + 1.29 b (ml)</td>
<td>35.2</td>
<td>0.815</td>
<td>3.26</td>
<td>**</td>
</tr>
<tr>
<td>Grass Silage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cb (l/h)</td>
<td>0.0275 - 0.000034 DM (g/kg) + 0.000034 NDF (g/kgDM) + 0.463 cb (l/h)</td>
<td>0.076</td>
<td>0.813</td>
<td>0.0068</td>
<td>***</td>
</tr>
<tr>
<td>Lag (h)</td>
<td>-8.00 + 0.0183 DM (g/kg) + 0.00766 NDF (g/kgDM) + 0.781 lag (h)</td>
<td>5.18</td>
<td>0.79</td>
<td>0.87</td>
<td>**</td>
</tr>
<tr>
<td>Maize Silage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (ml)</td>
<td>5.34 + 1.01 a (ml) - 0.0281 DM (g/kg) + 0.0003 NDF (g/kgDM) + 0.0928 VFAcid (g/kgDM)</td>
<td>10.6</td>
<td>0.918</td>
<td>1.88</td>
<td>***</td>
</tr>
<tr>
<td>Ca (l/h)</td>
<td>0.2</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B (ml)</td>
<td>34.4 + 0.703 b (ml) - 0.0280 NDF (g/kgDM) - 0.120 VFAcid (g/kgDM)</td>
<td>56.9</td>
<td>0.733</td>
<td>3.72</td>
<td>**</td>
</tr>
<tr>
<td>Cb (l/h)</td>
<td>0.062 - 0.000024 DM (g/kg) - 0.000063 NDF (g/kgDM) + 0.481 cb (l/h)</td>
<td>0.072</td>
<td>0.821</td>
<td>0.0033</td>
<td>***</td>
</tr>
<tr>
<td>Lag (h)</td>
<td>-1.01 + 1.24 lag (h) - 0.00544 DM (g/kg) + 0.00394 NDF (g/kgDM)</td>
<td>5.05</td>
<td>0.951</td>
<td>0.27</td>
<td>***</td>
</tr>
<tr>
<td>Wholecrop Wheat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (ml)</td>
<td>2.92 + 1.11 a (ml) - 0.00615 DM (g/kg)</td>
<td>8.99</td>
<td>0.765</td>
<td>2.21</td>
<td>**</td>
</tr>
<tr>
<td>Ca (l/h)</td>
<td>0.2</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B (ml)</td>
<td>-6.5 + 1.2 b (ml) - 0.0091 DM (g/kg) + 0.145 VFAcid (g/kgDM)</td>
<td>56.9</td>
<td>0.844</td>
<td>5.28</td>
<td>**</td>
</tr>
<tr>
<td>Cb (l/h)</td>
<td>0.0038 + 0.842 cb (l/h) - 0.000025 DM (g/kg) + 0.000215 VFAcid (g/kgDM)</td>
<td>0.057</td>
<td>0.838</td>
<td>0.0075</td>
<td>**</td>
</tr>
<tr>
<td>Lag (h)</td>
<td>0.201 + 0.967 lag (h)</td>
<td>5.37</td>
<td>0.942</td>
<td>0.49</td>
<td>***</td>
</tr>
</tbody>
</table>

NS: P>0.05  *P<0.05  **P<0.01  ***P<0.001
3.5 DISCUSSION

3.5.1 EXPERIMENT 1 – EFFECT OF PARTICLE SIZE ON THE CARBOHYDRATE DEGRADATION PARAMETERS OF DRIED AND MILLED FORAGES ESTIMATED USING IVGPT

The routine milling of samples through a 1mm sieve results in particles that are significantly less than 1mm with only 0.2-0.5% of particles greater than 1mm and the majority less than 0.5mm. There was no significant effect of silage type on the distribution of particles less than 1mm (P>0.05) which indicates that milling effectively destroys any physical characteristics of the original silage that might promote chewing activity or mediate microbial fermentation by restricting the surface area available for microbial colonisation (Hanna et al., 1973). The mean particle size of the silages after milling was 0.18-0.21mm which is significantly smaller than the sieve aperture and smaller than the critical size proposed by Poppi et al., (1981). Failure of the 1mm sieve to produce particles closer to 1mm in size is most probably due to the sieve not actively cutting the dried samples but acting as an abrasive surface against which the dried material simply shatters.

Although the sieving technique has been commonly used to assess the particle size of dietary rations (Kononoff, 2002) and rumen and faecal contents (Poppi et al., 1981, Dixon and Milligan, 1985; Cordoza and Mertens, 1986; Shaver et al., 1988; Grant et al., 1990; Beauchemin et al., 1994) it was assumed in this study that the technique could also be used to measure the particle size distribution of dried and milled material. However, without microscopic assessment of the particles retained on each sieve, for which the necessary equipment was unavailable, the size and distribution of dried and milled particles can only be predicted using the sieving technique and may be inaccurate.

The shear strength of plant components is dependent on the concentration of NDF (Hughes et al., 2000) and for this reason it has been proposed that different plant components such as the leaf blades, leaf stems and grains may shatter differently upon grinding (Emanuele and Staples, 1988). This means that the different particle sizes isolated by sieving milled samples may represent different parts of the plant, and consequently the gas production profile of the different particle sizes may not only reflect the effect of particle size on gas production, but also the plant components found in each particle size (Emanuele and Staples, 1988).
There was no significant difference in the total gas produced by the different grass silage particle sizes (P>0.05), which indicates that the grass silage particles had a similar chemical composition and were equally digestible. However, the rate of fermentation of available carbohydrate was shown to be significantly affected by particle size, with the smallest grass silage particles (<0.25mm) shown to be more quickly fermented and producing significantly more QCHO attributed gas compared to larger particles. This is in agreement with other authors who attributed this to an increase in the level of cell contents released from finely ground particles (Nagadi, 1999; Valentin et al., 1999). Because small particles also provide a larger surface area for microbial fermentation, particle size was also shown to affect the rate of fermentation of SCHO, such as structural fibre, with a significant increase in the rate of SCHO attributed gas production as particle size decreased (P<0.05).

In contrast to grass silage, the total gas produced by maize silage and wholecrop wheat particles was significantly affected by particle size, indicating that plant material is not uniformly distributed across all particle sizes in these forages. This is not surprising considering that grass cut for silage is predominantly leafy material, whilst maize silage and wholecrop wheat is more heterogeneous containing leaf, stem, cob and grain.

Total gas production significantly increased as the particle size of maize silage and wholecrop wheat decreased (P<0.05), specifically in the level of gas production attributed to SCHO (P<0.05). A large component of SCHO attributed gas produced by maize silage and wholecrop wheat will be due to starch which although highly digestible, is only digested after an intial lag period (Chesson and Forsberg, 1988). This starch is contained in the cereal grain which, because of its low NDF concentration, will also have a lower shear strength (Hughes, et al., 2000) and will therefore be more susceptible to particle breakdown during milling. Consequently, a high proportion of small maize silage and wholecrop wheat particles (<0.5mm) may be starch particles, which would explain why smaller particles also had a shorter lag (P<0.05) and a higher volume of total (P<0.05) and SCHO attributed gas production (P<0.05) compared to larger particles, which would be assumed to contain a greater proportion of less digestible structural carbohydrates.
3.5.2 EXPERIMENT 2 – ESTIMATING THE CONTRIBUTION OF WATER SOLUBLE CARBOHYDRATES TO THE QCHO FRACTION OF SILAGE DURING IVGPT

Although this study indicates that particle size has a profound impact on the contribution of QCHO during rumen fermentation, the concentration of QCHO is also dependent on the fermentation of soluble plant components, which are more rapidly fermented than insoluble material (Chesson and Forsberg, 1988; Stefanon et al., 1996).

In measuring the kinetics of rumen degradation using nylon bags it is assumed that only soluble carbohydrates are quickly fermented, but this study shows that soluble carbohydrates only account for 80.3% of QCHO and 19.7% can be attributed to insoluble carbohydrates (Table 3.5). The nylon bag technique also assumes that soluble carbohydrates are immediately and completely degraded, but the results of this study indicate that soluble carbohydrate can also be slowly fermented, contributing 8.5% to the SCHO fraction (Table 3.5). These results are in agreement with Stefanon et al., (1996) who showed that some soluble components of Alfalfa and Brome hay are slowly fermenting and not immediately degraded.

Because soluble plant components are not all immediately degraded in the rumen it is also incorrect to describe the quickly degradable fraction as soluble and the slowly degradable fraction as insoluble. The advantage of using IVGPT is that soluble and insoluble plant components are equally likely to fermented, but the disadvantage is that the effects of rumen flow, which are crucial in estimating ration utilisation, are not considered. Soluble feed components in the liquid phase of rumen passage, pass out of the rumen (Aaes and Kristensen, 1997) significantly faster than those in the solid phase and have a greater chance of leaving the rumen undegraded (Aufrère et al., 2001). Feed solubility is therefore an important factor when formulating diets using IVGPT estimated degradation parameters, especially since soluble carbohydrates can be slow fermented (Table 3.5).

3.5.3 EXPERIMENT 3 – COMPARISON OF THE CARBOHYDRATE FERMENTATION PARAMETERS OF FRESH AND DRIED SILAGES ESTIMATED USING IVGPT

Forages are commonly dried and milled prior to in vitro incubation to improve handling and to produce a homogenous sample. In addition to the morphological effects of heat drying, the particle size of these milled forages
is significantly smaller (<0.5mm) (Table 3.1) than those commonly found in the rumen (>3-4.8mm) (Dixon and Milligan, 1981; Dixon and Milligan, 1985; Cordoza, 1985; Cordoza and Mertens, 1986; Shaver et al., 1988; Grant et al., 1990; Beauchemin et al., 1994). Analysing samples after they have been dried and milled may over or underestimate some degradation parameters of the forages when fed fresh.

Results showed that TCHO was 19.9% higher in fresh grass silage (P<0.001), 6.9% higher in fresh maize silage (P<0.01) and 14.9% higher in fresh wholecrop wheat (P<0.01) (Table 3.7), which is in agreement with other authors who also showed that dried and milled material was less degradable than fresh (Broesder et al., 1992; Bonsi et al., 1995; Nagadi et al., 1998; Valentine et al., 1999). This decline in silage degradability after drying is probably primarily due to the loss of volatile components (Snyman and Joubert, 1992; Denium and Maassen, 1994), as these make up a large proportion of the digestible fraction in fresh silages (Pichard and Van Soest, 1977).

In addition to a significant effect on TCHO, drying and milling also had a significant effect on the concentration of QCHO and SCHO (Table 3.7)

Although there was no significant difference in the concentration of SCHO (P>0.05), QCHO was 36.9% higher (P<0.001) in fresh grass silage compared to the dried material, indicating that the reduction in concentration of TCHO in grass silage after heat drying is principally due to a reduction in the level of QCHO rather than SCHO. These findings are supported by other others who have shown that heat drying reduces the digestibility of carbohydrates in the rumen by altering structural (Van Soest, 1982) and non-structural carbohydrates (Acosta and Kothmann, 1978; Piccaglia and Galleti, 1987; Ljókel et al., 2003) and the solubility of silage carbohydrates (Valentin et al., 1999) that are an important component of QCHO in fresh material.

In contrast to grass silage, there was no significant difference in the concentration of QCHO (P>0.05) in fresh maize silage or wholecrop wheat compared to the dried material, but SCHO was 6.7% (P<0.01) higher in fresh maize silage and 15.7% higher in fresh wholecrop wheat (P<0.01) (Table 3.7). These results indicate that the reduction in TCHO of maize silage and wholecrop wheat after heat drying is primarily due to a reduction in the concentration of SCHO, rather than QCHO. A large proportion of the SCHO in maize silage and wholecrop wheat is starch which is very susceptible to heat which causes morphological changes to its structure. Heat treated
cereal grain has been shown to be more resistant to microbial degradation and to promote an increase in the level of by-pass starch (Ljókel et al., 2003). The reduction in SCHO in these forages is therefore probably related to a reduction in the degradability of starch due to heat induced changes in its morphological structure.

Although the level of TCHO was significantly higher in fresh forage material the rate of fermentation of SCHO was 13.6% lower in fresh grass silage (P<0.01) and 12.5% lower (P<0.001) in fresh maize silage compared to dried material. These findings are supported by other authors who also showed that the rate of fermentation of fresh forages was lower than when dried and milled (Bonsi et al., 1995; Cone, 1998; Lowman et al., 2002). The lower rate of fermentation of SCHO in fresh material is probably related to the larger particle sizes found in the fresh material and the smaller surface area available for microbial fermentation compared to dried and milled material.

There was no significant difference in the lag between fresh and dried forages (P>0.05), indicating that the rate of microbial attachment of SCHO is not affected by drying and is no different to that observed for fresh forages.

3.5.3.1 COMPARISON OF THE CARBOHYDRATE FERMENTATION PARAMETERS OF DIFFERENT SILAGE TYPES

The relationship between the fermentation parameters of the different silage types was not significantly different when using dried or fresh material (P>0.05) (Table 3.8).

Grass silage was shown to have the highest concentration of QCHO compared to maize silage and wholecrop wheat, probably because the grass storage polysaccharide fructan is fermented more quickly in the initial stages of incubation than starch in maize silage and wholecrop wheat (McGrath, 1988), which contributes mainly to the SCHO fraction. For this reason the concentration of SCHO was also significantly higher in maize silage and wholecrop wheat compared to grass silage (P<0.05). However, maize silage was shown to have the highest concentration of SCHO presumably because the structural fibre of maize silage is more digestible than that of wholecrop wheat, which contained more acid detergent fibre (ADF) (Table 3.6).

Although the lag time was not significantly different between the different forage types, SCHO fermented significantly quicker in grass silage and maize silage compared to wholecrop wheat (P<0.05).
The concentration of TCHO was shown to be highest in maize silage compared to grass silage and wholecrop wheat (P<0.05), which were not significantly different (P>0.05). These results indicate that although grass silage and wholecrop wheat provide the same quantity of TCHO they have very different fermentation profiles. This highlights the benefits of using IVGPT to measure the kinetics of carbohydrate degradation as it provides the additional information required to optimise nutrient availability whilst also maintaining rumen function by managing the rate of carbohydrate fermentation.

3.5.3.2 PREDICTING THE CARBOHYDRATE FERMENTATION PARAMETERS OF FRESH SILAGES USING DRIED MATERIAL

Multiple linear regression equations were successfully developed to predict the gas production parameters of fresh forages from the gas production parameters of the same forages when dried and milled (Table 3.9). This enables data derived from dried and milled samples to be used in rumen models to predict the degradation profile of the forage when fed fresh. It also removes the need to use fresh material which is difficult to chop and tends to produce a heterogeneous sample which decreases the accuracy of measurement (Lopez et al., 1995).
3.6 CONCLUSION

The particle sizes of dried forages milled through a 1mm sieve are significantly less than 1mm with the majority of particles less than 0.5mm. The rate of fermentation significantly increases as particle size decreases, but in wholecrop cereals the interpretation of fermentation profiles is difficult because different particle sizes represent different parts of the plant, with the majority of small particles comprising of starch particles.

Not all soluble carbohydrate is quickly fermented in the rumen and a proportion of QCHO can be attributed to insoluble carbohydrates.

Fresh forages contained significantly more TCHO than dried material principally due to a higher concentration of QCHO in fresh grass silage and a higher concentration of SCHO in fresh maize silage and wholecrop wheat. There was no significant effect on the lag, but SCHO fermented significantly slower in fresh grass silage and fresh maize silage compared to dried material.
The effect of ammonia availability on the degradation profile of pure carbohydrates during IVGPT
Feed samples are usually incubated in a nitrogen rich environment during IVGPT to ensure that microbial fermentation is not limited by nitrogen availability. The purpose of this investigation was to study the effect of ammonia concentration on the gas production profile of pure carbohydrates during IVPGT and to assess the possibility of using IVGPT to measure nitrogen utilisation.

In addition to a positive linear relationship between gas production and the quantity of incubated carbohydrate (sucrose: $R^2=98.7$ $P<0.001$; maize starch: $R^2=99.5$ $P<0.001$; and glucose: $R^2=98.7$ $P<0.001$) there was also a negative linear relationship with the final nitrogen level (sucrose: $R^2=98.3$ $P<0.001$; maize starch: $R^2=99.9$ $P<0.001$) indicating that cumulative gas production can be related to nitrogen utilisation.

IVGPT was sensitive to nitrogen availability but the rate and volume of gas production was shown to be dependent on both the concentration of incubated nitrogen and the quantity and type of carbohydrate.

Although there was no significant difference in the efficiency of nitrogen utilisation between the different carbohydrate sources ($P>0.05$), the rate of gas production was significantly higher and sustained whilst incubating sucrose or glucose ($P<0.05$) which also had significantly higher utilisation efficiencies compared maize starch ($P<0.05$).

IVGPT was most sensitive to nitrogen availability when the incubated carbohydrate to nitrogen ratio was 42:1, up to maximum of 78.4mgN/l. At ammonia concentrations less than 78.4mgN/l there was a significant positive relationship between gas production and nitrogen availability, but above 78.4mgN/l gas production was shown to decrease ($P<0.05$). These results indicate that elevated rumen ammonia concentrations can have a detrimental effect on microbial activity at concentrations considerably lower than that which causes ammonia toxicity in the ruminant animal.

The conclusion to this study is that IVGPT is sensitive to nitrogen availability when the incubated sucrose to nitrogen ratio is 42:1 and where the availability of nitrogen is no more than 78.4mgN/l. This presents the possibility of using IVGPT to measure the kinetics of feed protein degradation but only on the proviso that a technique can be developed to remove ammonia nitrogen from rumen fluid with no detrimental effect on microbial activity.
4.2 INTRODUCTION

Ruminal microbes not only provide ruminants with the means of digesting fibrous plant material but they are also an important source of high quality protein (Clark et al., 1992; AFRC, 1993). The rate of microbial protein synthesis (MPS) and rumen digestion is highly dependent on the quantity and type of energy and protein available in the rumen. The low efficiency of nitrogen capture associated with high protein diets (Givens and Rulquin, 2003) is significantly improved by including readily fermentable carbohydrate such as soluble sugars (Chamberlain et al., 1985; Chamberlain et al., 1993). The synchronisation of ruminally available carbohydrate and protein is therefore key to optimise microbial growth, diet utilisation and absorbed microbial protein.

Rumen microbes derive the nitrogen required for maintenance and growth from a range of sources but the main source is ammonia, especially for microbes digesting cellulose and hemicellulose (Russel et al., 1992; Chenost and Kayouli, 1997). The concentration of ammonia in the rumen can have a significant influence on MPS which has been shown to be limited at ammonia concentrations below 20mgN/l (Satter and Slyter, 1974) and maximised at 50-88 mgN/l (Hoover, 1986; Hume et al., 1970; Satter and Slyter, 1974). Unfortunately, determining the optimum level of rumen ammonia required to maximise MPS is difficult when nitrogen is not limited because MPS is directly related to the quantity and type of carbohydrate fermented (Hungate, 1966; Erdman et al., 1986)

In IVGPT, the relationship between carbohydrate availability and nitrogen requirement was first highlighted by Raab et al., (1983) who showed that in addition to a positive linear relationship between gas production and quantity of incubated carbohydrate (starch) there was also a corresponding linear decline in the ammonia concentration of the rumen fluid. This observation was further supported by Nagadi (1999) who demonstrated that the nitrogen required to maximise the rate of gas production increased as the concentration of digestible cell wall content of the substrate increased.

Because feed protein liberated during incubation is used in part for microbial protein synthesis, it had been suggested that the rate and quantity of gas production during IVGPT can also be regarded as a measure of the nitrogen available for MPS (Raab et al., 1983). Further investigation also showed that the rate of gas production during IVGPT was sensitive to nitrogen
availability (Raab et al., 1983; Nagadi, 1999). This was attributed to the fact that when nitrogen is limiting, MPS decreases and a greater proportion of carbohydrate is used to fuel microbial maintenance resulting in a drop in gas production.

This assumption was supported by Krishnamoorthy et al., (1991) who observed a linear relationship between MPS and the cumulative gas production of a carbohydrate mixture in the first 8 hours of incubation. Although the efficiency of MPS after 8 hours was shown to be subsequently dependent on the rate of carbohydrate fermentation and nitrogen availability (Russel et al., 1992) these findings suggest the possibility of using cumulative gas production during IVGPT as an index of MPS and using it to determine the optimum rumen ammonia level required to maximise the rate of fermentation of pure substrates.

The objectives of this study were to determine the carbohydrate fermentation profiles of sucrose, glucose and maize starch during IVGPT and to 1) determine if gas production is sensitive to nitrogen availability, 2) determine the rumen ammonia concentration required to optimise carbohydrate fermentation and 3) determine the concentration of carbohydrate and ammonia required to maximise the rate of gas production during IVGPT.
4.3 MATERIAL AND METHODS

4.3.1 EXPERIMENTAL AIM

The experiment was designed to study the effect of nitrogen availability on the gas production parameters of pure carbohydrate substrates during IVGPT and to determine the optimum concentration of carbohydrate and ammonia required to maximise gas production.

The overall design was to incubate three quantities (50mg, 100mg and 150mg) of sucrose, glucose and maize starch in buffered rumen fluid with three distinct ammonia concentrations (54.8, 78.4, 102.1 mgN/l)

4.3.2 ESTIMATING THE RUMEN FERMENTATION USING IVGPT

The appropriate weights (50mg, 100mg and 150mg) of sucrose, maize starch and glucose were weighed in duplicate into 100 ml glass syringes and stored at room temperature until the morning of incubation. For each ammonia concentration three ‘blank’ syringes were also created and stored at room temperature but contained no substrate.

On the morning of inoculation and prior to collecting the rumen liquor, the syringes containing the samples, along with ‘blanks’ containing no substrate, were placed in a 39°C water bath.

Rumen liquor was collected from a fistulated beef steer fed a ration of 70% grass hay and 30% pellets. The steer was starved for 12 hours prior to collection. On the morning of inoculation, rumen contents was removed from the rumen, gently squeezed and the rumen liquor collected in a warmed thermos flask to keep warm whilst travelling back to the laboratory. At the laboratory the rumen liquor was strained through two layers of 250 µm netting and three layers of muslin, and then stored in a thermos flask gassed with CO₂.

To ensure an adequate microbial population is incubated with each sample and to enable comparison of samples in different gas production runs, microbial activity of the rumen liquor was assessed by measuring its absorbance at 600 nm (Nagadi et al., 2000). The dilution ratio with anaerobic medium was then calculated to ensure that the inoculum had an absorbance
at 600 nm of 0.111 - 0.134, thereby ensuring that microbial activity among the IVGPT runs was similar.

The appropriate volume of anaerobic buffer medium (ABM) (Menke and Steingass, 1988) was prepared in three 1.5l round bottom flasks using three buffer solutions with different concentrations of ammonia (B1, B2, B3), place in a 39°C water bath and saturated with CO₂:

B1 71 mgN/l (0.4g NH₄NCO₃ + 38.6g NaHCO₃ in 1L of distilled H₂O)
B2 106 mgN/l (0.6g NH₄NCO₃ + 38.4g NaHCO₃ in 1L of distilled H₂O)
B3 142 mgN/l (0.8g NH₄NCO₃ + 38.2g NaHCO₃ in 1L of distilled H₂O)

When each ABM had reached 39°C, rumen liquor was added to the ABM made with B1 (ABMB1), gassed with CO₂ for 5 minutes and sealed with sponge and cling film. This procedure was repeated with ABMB2 and ABMB3.

The clinging film was removed from the ABMB1 inoculum which was gassed with CO₂ for 5 minutes. Then using an automatic dispensing pump, 30 ml of inoculum was added to all relevant fermentation syringes which were read and shaken before being returned to the water bath. This procedure was repeated for the ABMB2 and ABMB3 inocula. Measurement of in vitro gas production were performed as described by Jessop and Herrero (1996), with cumulative gas production in each syringe read every half hour for the first 3 hours, then hourly until 6 hours and thereafter at 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44 and 48 hours. Syringes were manually shaken at each reading.

4.3.3 INTERPRETATION OF GAS PRODUCTION DATA

The fermentation profile of each syringe was determined by applying the analytical procedure described in Appendix A1.4. This describe forage fermentation in terms of the volume and rate of gas production attributed to QCHO and then after a lag, the volume and rate of gas production attributed to SCHO.

4.3.4 MEASUREMENT OF THE AMMONIA CONCENTRATION OF THE INOCULUM

The ammonia concentration of the inoculum before and after 48 hours of incubation was measured using an ammonia probe.
4.3.5 CALCULATION OF CARBOHYDRATE AND NITROGEN UTILISATION EFFICIENCIES

The carbohydrate and nitrogen utilisation efficiency was calculated assuming that all the available carbohydrate was degraded in the 48 hour incubation period:

\[
\text{Carbohydrate Utilisation Efficiency (ml/mg)} = \frac{\text{Total Gas volume at 48 hours (ml)}}{\text{sample weight (mg)}}
\]

\[
\text{Nitrogen Utilisation Efficiency (ml/mgN)} = \frac{\text{Total gas volume at 48 hours (ml)}}{((\text{Initial (mgN/l)} - \text{Final (mgN/l)}) \times (30 / 1000))}
\]

4.3.6 STATISTICAL ANALYSIS

Using the MINITAB statistical package (2000), paired T-tests were used to compare the gas production parameters of the different carbohydrate weights and ammonia concentration. Analysis of variance (ANOVA) using a general linear model (GLM) was used to compare the difference between different carbohydrate types. Linear regression was used to describe the relationship between the weight of carbohydrate incubated, the total volume of gas produced and the nitrogen level of the inoculum after the 48 hour incubation.
4.4 RESULTS

4.4.1 EFFECT OF CARBOHYDRATE AND AMMONIA CONCENTRATION ON THE GAS PRODUCTION PROFILE AND EFFICIENCY OF UTILISATION OF SUCROSE, MAIZE STARCH AND GLUCOSE DURING IVGPT

The gas production profiles of each carbohydrate are illustrated in Fig 4.1, Fig 4.2 and Fig 4.3 and the relationship between gas production, carbohydrate weight and nitrogen utilisation presented in Table 4.1. The relationship between carbohydrate weight and final ammonia concentration is illustrated for sucrose and maize starch in Fig 4.4. The utilisation efficiency of each carbohydrate during incubation is presented in Table 4.2 and illustrated in Fig 4.5, Fig 4.6 and Fig 4.7.

There was a positive linear relationship between the volume of gas produced after 48 hours and the weight of incubated sucrose ($R^2=98.7$, $P<0.001$), maize starch ($R^2=99.5$, $P<0.001$) and glucose ($R^2=98.7$, $P<0.001$). In addition, there was also a negative linear relationship shown between the weight of incubated carbohydrate and the final ammonia concentration when incubating sucrose ($R^2=-98.3$, $P<0.001$) and maize starch ($R^2=-99.9$, $P<0.001$).

The volume of gas produced during the 48 hour incubation and the subsequent efficiency of carbohydrate utilisation by rumen microbes (ml/mg of carbohydrate) was significantly affected by the ammonia concentration.

During incubation of 50 and 100mg of each carbohydrate type, there was a significant increase in the efficiency of carbohydrate utilisation when the ammonia concentration increased from 54.8mgN/l to 78.4mgN/l ($P<0.05$), but no further increase in efficiency when the ammonia concentration was increased from 78.4mgN/l and 102.1mgN/l ($P>0.05$).

The ammonia concentration had no significant effect on the efficiency of carbohydrate utilisation when 150mg of each carbohydrate was incubated ($P>0.05$).

For all the carbohydrate types, the highest efficiency of carbohydrate utilisation was shown to be during the incubation of 100mg of carbohydrate in rumen liquor with an ammonia concentration of 78.4mgN/l.
**Fig 4.1** Fitted cumulative gas production of 50mg (grey), 100mg (black) or 150mg (white) of sucrose incubated in duplicate (n=2) in bovine rumen liquor with an ammonia concentration of 54.8 mgN/l (circle), 78.4 mgN/l (square) or 102.1 mgN/l (triangle). Cumulative gas production curves were fitted to the equation: $\text{Gas} = A(1-\exp(-ctd))+B(1-\exp(-c(t-Lag)))*\exp(5(t-Lag))/(1+\exp(5(t-Lag)))$ modified from Jessop and Herrero (1996) [see text for interpretation of parameters].

**Fig 4.2** Fitted cumulative gas production of 50mg (grey), 100mg (black) or 150mg (white) of maize starch incubated in duplicate (n=2) in bovine rumen liquor with an ammonia concentration of 54.8 mgN/l (circle), 78.4 mgN/l (square) or 102.1 mgN/l (triangle). Cumulative gas production curves were fitted to the equation: $\text{Gas} = A(1-\exp(-ctd))+B(1-\exp(-c\alpha(t-Lag)))*\exp(5(t-Lag))/(1+\exp(5(t-Lag)))$ modified from Jessop and Herrero (1996) [see text for interpretation of parameters].
Fig 4.3 Fitted cumulative gas production of 50mg (grey), 100mg (black) or 150mg (white) of glucose incubated in duplicate (n=2) in bovine rumen liquor with an ammonia concentration of 54.8 mgN/l (circle), 78.4 mgN/l (square) or 102.1 mgN/l (triangle). Cumulative gas production curves were fitted to the equation: $\text{Gas} = A(1-\exp(-ct)) + B(1-\exp(-ct(t-Lag)))/\exp(5(t-Lag))/\exp(5(t-Lag))$ modified from Jessop and Herrero (1996) [see text for interpretation of parameters].

Table 4.1 Linear relationship between carbohydrate weight, total gas production at 48 hours and the final ammonia concentration when sucrose, maize starch and glucose were incubated in duplicate (n=2) in bovine rumen liquor with an ammonia concentration of 78.4 mgN/l.

<table>
<thead>
<tr>
<th>Carbohydrate Type</th>
<th>Equation</th>
<th>$R^2$</th>
<th>SEE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gas Production in 48 hours (ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sucrose</em></td>
<td>$1.48 + (0.380*\text{sucrose (mg)})$</td>
<td>98.7</td>
<td>2.21</td>
<td>***</td>
</tr>
<tr>
<td><em>Maize Starch</em></td>
<td>$0.14 + (0.386*\text{maize starch (mg)})$</td>
<td>99.5</td>
<td>1.43</td>
<td>***</td>
</tr>
<tr>
<td><em>Glucose</em></td>
<td>$0.99 + (0.357*\text{glucose (mg)})$</td>
<td>98.7</td>
<td>2.04</td>
<td>***</td>
</tr>
<tr>
<td><strong>Final ammonia level (mgN/l)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sucrose</em></td>
<td>$48.3 - (0.255*\text{sucrose (mg)})$</td>
<td>-98.3</td>
<td>1.66</td>
<td>***</td>
</tr>
<tr>
<td><em>Maize Starch</em></td>
<td>$52.0 - (0.311*\text{maize starch (mg)})$</td>
<td>-99.9</td>
<td>0.35</td>
<td>***</td>
</tr>
</tbody>
</table>

NS $P>0.05$ *$P<0.05$ **$P<0.01$ ***$P<0.001$
Fig 4.4 Linear relationship between final nitrogen concentration (mgN/l) and the weight of sucrose and maize silage incubated using IVGPT in bovine rumen liquor with an initial nitrogen concentration of 78.4 mgN/l

Table 4.2 The effect of carbohydrate and nitrogen concentration on the utilisation efficiency of sucrose, glucose and maize starch (ml/mg) by rumen microbes during IVGPT. 50mg, 100mg and 150mg of each carbohydrate were incubated for 48 hours in bovine rumen liquor with an ammonia concentration of either 54.8 mgN/l, 78.4 mgN/l or 102.1 mgN/l. Each carbohydrate was incubated in duplicate (n=2). The utilisation efficiencies of sucrose was adjusted for the molar availability of carbon when compared to glucose. For each carbohydrate weight, means with different superscripts within the same row are significantly different (P<0.05).

<table>
<thead>
<tr>
<th>Carbohydrate Type</th>
<th>Weight (mg)</th>
<th>54.8</th>
<th>78.4</th>
<th>102.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>50</td>
<td>0.307&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.369&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.365&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.359&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.394&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.377&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.360</td>
<td>0.364</td>
<td>0.369</td>
</tr>
<tr>
<td>Glucose</td>
<td>50</td>
<td>0.295&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.358&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.355&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.351&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.385&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.370&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.354</td>
<td>0.357</td>
<td>0.362</td>
</tr>
<tr>
<td>Maize Starch</td>
<td>50</td>
<td>0.318&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.378&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.373&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.340&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.398&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.350&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0.345</td>
<td>0.383</td>
<td>0.378</td>
</tr>
</tbody>
</table>
**Fig 4.5** The effect of carbohydrate and nitrogen concentration on the microbial utilisation efficiency (ml gas/mg) of sucrose during IVGPT adjusted for the molar availability of carbon when compared to glucose. 50mg, 100mg and 150mg of sucrose was incubated in duplicate (n=2) for 48 hours in bovine rumen liquor with an ammonia concentration of either 54.8 mgN/l, 78.4 mgN/l or 102.1mgN/l.

**Fig 4.6** The effect of carbohydrate and nitrogen concentration on the microbial utilisation efficiency (ml gas/mg) of glucose during IVGPT. 50mg, 100mg and 150mg of glucose was incubated in duplicate (n=2) for 48 hours in bovine rumen liquor with an ammonia concentration of either 54.8 mgN/l, 78.4 mgN/l or 102.1 mgN/l.
Fig 4.7 The effect of carbohydrate and nitrogen concentration on the microbial utilisation efficiency (ml gas/mg) of maize starch during IVGPT. 50mg, 100mg and 150mg of maize starch was incubated in duplicate ($n=2$) for 48 hours in bovine rumen liquor with an ammonia concentration of either 54.8 mgN/l, 78.4 mgN/l or 102.1 mgN/l.

4.4.2 COMPARISON OF THE GAS PRODUCTION PROFILE AND UTILISATION EFFICIENCY OF SUCROSE, GLUCOSE AND MAIZE STARCH DURING IVGPT

The gas production profile and utilisation efficiencies of 100mg of sucrose, glucose and maize starch incubated in rumen inoculum with an ammonia concentration of 78.4mgN/l is presented in Table 4.3. The gas production profile and utilisation efficiencies of 100mg of sucrose and glucose corrected to equal molar quantities of carbon when incubated in rumen inoculum with an ammonia concentration of 78.4mgN/l is presented in Table 4.4.

Sucrose produced significantly more gas in the first 12 hours of incubation compared to maize starch ($P<0.05$) (Table 4.3) but the same volume of gas as glucose when adjusted for carbon availability ($P>0.05$) (Table 4.4). After 12 hours there was no significant difference in the volume of gas produced by maize starch, glucose or sucrose when adjusted for carbon availability ($P>0.05$).

Although there was no significant difference in the final ammonia concentration or efficiency of nitrogen utilisation between the different
carbohydrate types (P>0.05), maize starch had a significantly lower efficiency of carbohydrate utilisation compared to sucrose and glucose which when corrected for carbon availability were not significantly different (P>0.05).

**Table 4.3** Gas production parameters and utilisation efficiencies of 100 mg of sucrose, glucose and maize starch incubated in bovine ruminal fluid with a nitrogen concentration of 78.4mgN/l. Each value is the mean of two replicates (n=2). For each carbohydrate type, means with different superscripts within the same row are significantly different (P<0.05)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sucrose</th>
<th>Glucose</th>
<th>Maize Starch</th>
<th>Type Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vol @ 6h (ml)</td>
<td>20.12</td>
<td>18.64</td>
<td>7.89</td>
<td>***</td>
</tr>
<tr>
<td>Vol @ 12h (ml)</td>
<td>29.04</td>
<td>26.93</td>
<td>20.56</td>
<td>*</td>
</tr>
<tr>
<td>Vol @ 24h (ml)</td>
<td>37.58</td>
<td>34.88</td>
<td>37.33</td>
<td>*</td>
</tr>
<tr>
<td>Vol. @48h(ml)</td>
<td>41.54</td>
<td>38.48</td>
<td>39.82</td>
<td>*</td>
</tr>
<tr>
<td>Vol @ 6h (%Vol. @48h)</td>
<td>48.45</td>
<td>48.43</td>
<td>19.80</td>
<td>***</td>
</tr>
<tr>
<td>Vol @ 12h (%Vol. @48h)</td>
<td>69.91</td>
<td>69.97</td>
<td>51.63</td>
<td>***</td>
</tr>
<tr>
<td>Vol @ 24h (%Vol. @48h)</td>
<td>90.48</td>
<td>90.64</td>
<td>93.75</td>
<td>***</td>
</tr>
<tr>
<td>Final N (mgN/l)</td>
<td>21.00</td>
<td>-</td>
<td>20.90</td>
<td>NS</td>
</tr>
<tr>
<td>N Efficiency (ml/mgN)</td>
<td>24.12</td>
<td>-</td>
<td>23.09</td>
<td>NS</td>
</tr>
<tr>
<td>CHO Efficiency (ml/mgCHO)</td>
<td>0.415</td>
<td>0.385</td>
<td>0.398</td>
<td>*</td>
</tr>
</tbody>
</table>

NS P>0.05  *P<0.05  **P<0.01  ***P<0.001

**Table 4.4** Gas production parameters and utilisation efficiencies of sucrose and glucose adjusted for molar carbon availability, incubated in bovine ruminal fluid with a nitrogen concentration of 78.4mgN/l. Each value is the mean of two replicates (n=2). For each carbohydrate type, means with different superscripts within the same row are significantly different (P<0.05)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sucrose</th>
<th>Glucose</th>
<th>Type Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vol @ 6h (ml)</td>
<td>19.11</td>
<td>18.64</td>
<td>NS</td>
</tr>
<tr>
<td>Vol @ 12h (ml)</td>
<td>27.59</td>
<td>26.93</td>
<td>NS</td>
</tr>
<tr>
<td>Vol @ 24h (ml)</td>
<td>35.70</td>
<td>34.88</td>
<td>NS</td>
</tr>
<tr>
<td>Vol. @48h(ml)</td>
<td>39.46</td>
<td>38.48</td>
<td>NS</td>
</tr>
<tr>
<td>CHO Efficiency (ml/molCHO)</td>
<td>0.395</td>
<td>0.385</td>
<td>NS</td>
</tr>
</tbody>
</table>
4.5 DISCUSSION

4.5.1 EFFECT OF AMMONIA CONCENTRATION ON THE GAS PRODUCTION PARAMETERS AND UTILISATION EFFICIENCIES OF PURE CARBOHYDRATE SUBSTRATES DURING IVGPT

MPS is dependent on both the concentration of ammonia in the rumen (Satter and Slyter, 1974; Hume et al., 1970; Hoover, 1986) and the quantity and type of available carbohydrate (Chamberlain et al., 1985; Chamberlain et al., 1993). This synchronous relationship between carbohydrate and nitrogen availability has previously made it difficult to assess the optimum ammonia concentration required to maximise MPS (Hungate, 1966; Erdman et al., 1986).

Because the rate of gas produced during IVGPT is sensitive to nitrogen availability (Raab et al., 1983; Nagadi, 1999) with a linear relationship between gas production in the first 8 hours of incubation and MPS, the rate and quantity of gas production during IVGPT could be used as an index of MPS (Krishnamoorthy et al., 1991). This proposal is supported by this study which showed that the positive linear relationship between gas production and the quantity of incubated carbohydrate (sucrose: $R^2=98.7\, P<0.001$; maize starch: $R^2=99.5\, P<0.001$; and glucose: $R^2=98.7\, P<0.001$) was also associated with a linear decline in the final nitrogen concentration of sucrose ($R^2=98.3\, P<0.001$) and maize starch: $R^2=99.9\, P<0.001$). Unfortunately, the final nitrogen concentration when incubating glucose was not measured due equipment failure, but the attainable results indicate that cumulative gas production can be directly related to nitrogen utilisation at least when incubating sucrose or maize starch.

Assuming that the incubated carbohydrate is completely degraded after 48 hours, the total gas produced by each carbohydrate can then be used as an index of the efficiency of carbohydrate utilisation by the rumen microbes. So whilst increasing the inclusion of carbohydrate was associated with an increase in gas production, the concentration of ammonia was also shown to have a significant effect on the efficiency of carbohydrate utilisation. During incubation of 50mg and 100mg of each carbohydrate there was a significant increase in the efficiency of carbohydrate utilisation when the ammonia concentration increased from 54.8mgN/l to 78.4mgN/l($P>0.05$). It is possible that altering the ammonium concentration of the inoculum could change the ionic strength of the buffer. This would result in the production of more
indirect gas which would mean that any observed increase in gas production could not necessarily be attributed solely to an increase in microbial activity or efficiency. However, there was no further increase in gas production or efficiency when the ammonia concentration was increased to 102.1 mgN/l, indicating that ammonia concentration does indeed have a direct effect on microbial activity and not just on the ionic strength of the buffer. This was particularly true during incubation of 100 mg of each carbohydrate were there was a significant decline in the efficiency of carbohydrate utilisation when the ammonia concentration increased from 78.4 mgN/l to 102.1 mgN/l.

In addition to supporting previous studies which showed a beneficial effect of ammonia on gas production during IVGPT (Nagadi, 1999) and on MPS and digestibility in the rumen (Leng, 1990), these results indicate that elevated ammonia concentrations can also have a detrimental effect on microbial development and substrate fermentation. Although previous studies have shown that there is no beneficial advantage of increasing rumen ammonia above that which promotes the maximum substrate digestibility (Leng, 1990; Nagadi, 1999), no studies have observed a deleterious effect of high ammonia concentrations on microbial fermentation in the rumen. In contrast, high ammonia concentrations have been shown to reduce the methane production and chemical oxygen demand (COD) efficiency of methanogenic bacteria in anaerobic sludge fermenters (Lay et al., 1998; Sung and Lui, 2003) with an increase in ammonia concentration from 40 mgN/L to 120 mgN/L resulting in a 17% reduction in productivity (Sung and Lui, 2003).

Ammonia exists in the rumen in two forms: unionized (NH₃) and ionized (NH₄⁺). The state of equilibrium that exists between these two forms is dependent on the pH, ionic strength and temperature of the rumen fluid and it is these factors that determine the relative concentrations of ammonium ions (NH₄⁺), free ammonia (NH₃), hydrogen ions (H⁺) and hydroxyl ions (OH⁻). Free ammonia is capable of penetrating the cell membrane (Gallen and Winter, 1997) and at high concentrations has been suggested to induce free radical formation within the cell (Murthy et al., 2001) and promote mitochondrial permeability transition (MPT) (Rama et al., 2003). MPT is associated with the opening of pores on the inner mitochondrial membrane and this causes the collapse of the ionic gradient, mitochondrial dysfunction (Rama et al., 2003) and a reduction in ATP synthesis (Zwingmann and Leibfritz, 2005).
The results of this study indicate high ammonia concentrations may have a similar effect on microbial activity in the rumen, which in this study was significantly reduced whilst incubating 100mg of sucrose when the ammonia concentration increased form 78.4mgN/l to 102.1 mgN/l (P<0.05). However, because there was no significant difference in the utilisation efficiency of microbes in 78.4mgN/l and 102.1mgN/l when the concentration of carbohydrate was increased to 150mg (P>0.05) these results indicate that microbial resistance to high ammonia concentrations may be energy dependent. Providing extra carbohydrates may enable microbes to resist the effects of high ammonia concentrations by supplying the extra energy required either for direct removal of ammonia from the interior of the cell or for detoxification via conversion to glutamine (Murthy et al., 2001).

Because an ammonia concentration of 102.1mgN/l was associated with a decline in microbial activity, the results of this study indicate that the ammonia concentration required for maximum digestibility and MPS in the rumen is 78.4 mgN/l. This is significantly higher than the optimum ammonia concentration suggested by Satter and Slyter (1974) (50 mgN/l) but similar to that proposed by Hume et al., (1970) (88 mgN/l) and Hoover (1986) who proposed an optimum concentration of 80 mgN/l for nutrient digestion and 62 mgN/l for microbial growth and digestion. The results of this study also agree with Nagadi (1999) who although stating that the optimum ammonia concentration for the fermentation of cellulose during IVGPT was 105 mgN/l had miscalculated the culture concentration which was actually 68.3 mgN/l. The optimum rumen ammonia concentration for rumen microbes may therefore be significantly lower than that which has a toxic effect in ruminants (~300mgN/L).

### 4.5.2 MEASURING MPS USING IVGPT

The results of this study show that although IVGPT is sensitive to nitrogen availability it is most sensitive when the incubated carbohydrate to nitrogen ratio is 42:1 with a maximum nitrogen concentration of 78.4mgN/l. At this optimum carbohydrate to nitrogen ratio, microbes have the highest efficiency of carbohydrate utilisation and gas production is positively correlated to nitrogen availability. To ensure that any changes in gas production are within the accuracy of the technique it is imperative that the maximum quantity of carbohydrate is incubated, which in this study equated to 100mg of carbohydrate being incubated in 30ml of buffered rumen fluid with a nitrogen concentration of 78.4mgN/l.
Using IVGPT to estimate nitrogen availability also means that IVGPT could be used to measure the protein degradation characteristics of forages and feeds. However, before IVGPT can be developed to measure protein degradation a suitable carbohydrate source to support MPS must be chosen. It must be fermented quickly enough to enable rumen microbes to utilise any quickly fermenting protein (QDP) available in the first 6-7 hours of incubation, but also provide sufficient energy to enable microbes to utilise any slowly degradable protein (SDP) available in the first 20 hours of incubation (Cone and van Gelder, 1999).

Sucrose and glucose was shown to produce significantly more gas in the first 6 hours of IVGPT compared maize starch (P<0.05) (Table 5.3), indicating that they are a superior source of quickly fermentable carbohydrate (QCHO). After 24 hours, the volume of gas produced by sucrose and glucose was also a significantly smaller proportion of total gas production compared to maize starch (P<0.05), when corrected for the molar availability of carbon. This indicates that sucrose and glucose are also superior sources of carbohydrate to support SDP degradation.

There was no significant difference in the final nitrogen concentration or nitrogen utilisation efficiency between the different carbohydrate types (P>0.05), but the efficiency of utilisation was significantly lower in maize silage compared to sucrose and glucose, which after correction for molar carbon availability were not significantly different (P>0.05). Assuming that the VFA profile is similar between the different carbohydrate types, these results indicate that sucrose and glucose are more readily digested than maize starch and are therefore more likely to promote greater levels of MPS.

To measure nitrogen availability using IVPGT it is recommended that sucrose or glucose are incubated with nitrogen in a ratio of 42:1 with a maximum nitrogen concentration of 78.4mgN/l. However, before IVGPT can be used to estimate the protein degradation characteristics of feeds, a technique needs to be developed to remove all background nitrogen from the rumen fluid to ensure that the only nitrogen available to the rumen microbes is from feed protein.
IVGPT is sensitive to the availability of nitrogen and is most sensitive when the incubated carbohydrate to nitrogen ratio is 42:1 with a maximum nitrogen concentration of 78.4mgN/l.

Rumen ammonia levels greater than 78.4 mgN/L have a detrimental effect on the efficiency of microbial digestion and growth.

Sucrose and glucose are more readily digested than maize starch and are assumed to promote a higher level of MPS.

There is a linear relationship between total gas production and nitrogen utilisation which presents the possibility of using IVGPT to measure the kinetics of feed protein degradation.
Ammonium absorbance capacity of zeolite during column filtration and the effects of filtration on microbial activity during IVGPT
Because IVGPT is sensitive to nitrogen availability it has been proposed that it could be adapted to measure the kinetics of feed protein degradation in the rumen. However, any use of IVGPT in measuring feed protein degradation is dependent on the feed samples being incubated in a nitrogen free environment. The purpose of this investigation was to study the ammonium absorption capacity of zeolite during column filtration and to assess the effect of filtration of rumen liquor on microbial activity estimated using IVGPT.

During column filtration, the ammonium absorption coefficient of zeolite is significantly reduced as the bed volume flow rate is increased (P<0.001). However at flow rates greater than 68.2 BV/h there was no further decrease in the absorption coefficient (P>0.05), indicating that zeolite has a minimum absorption coefficient of ~1mgNH₃-N/g zeolite.

The optimum practical bed volume flow rate is 8.93 BV/h which equates to an absorption coefficient of 2.95mgNH₃-N/g zeolite. The effect of flow rate on the absorption coefficient of zeolite can be predicted using a 3-phase hyperbolic decay curve(R²=0.998, P<0.001).

Filtration of rumen liquor through zeolite had no significant effect on the rumen bacterial population estimated from its absorbance at 600nm (P>0.05) but it significantly increased microbial performance when fermenting sucrose during IVGPT. Filtered rumen liquor produced 2.9% more gas in the first 6 hours (P<0.05), had a shorter lag (P<0.001), produced 7.9% more slow gas (B fraction) (P<0.001) and 1.7% more total gas (P<0.01) compared to unfiltered rumen liquor. The superior microbial activity in the filtered rumen liquor may be related to selective retention of protozoa in the zeolite column.
5.2 INTRODUCTION

Because IVGPT has been shown to be sensitive to nitrogen availability (Raab et al., 1983; Nagadi, 1999) it has been proposed that IVGPT could be used to measure the kinetics of feed protein degradation (see Chapter 4). Using IVGPT to measure the utilisation of feed protein by rumen microbes is based on the principle that the only nitrogen available to the microbes will be from the incubated feed. This means that any background nitrogen present in the rumen liquor needs to be removed prior to incubation. Nagadi (1999) successfully reduced the ammonia concentration of rumen liquor to 5mgN/l by using the centrifugation techniques of Henning et al., (1991) and Pell and Schofield (1993) to isolate microbial bacteria, fungi and protozoa and then re-suspending them in nitrogen free medium. Although this approach had no significant effect on microbial activity (Nagadi, 1999), the centrifugation and washing techniques employed are time consuming and result in the microbes being aerobic and below 39°C for a considerable period of time.

Microbial degradation of feed protein in the rumen results in the production of ammonia, which is the main source of nitrogen for most rumen microbes (Russel et al., 1992; Chenost and Kayouli, 1997). Depending on pH, ionic strength and temperature of the surrounding solution ammonia can exist in either an unionized (NH₃) or ionized (NH₄⁺) form, which in rumen fluid means most ammonia is present as ammonium (NH₄⁺) (Koo et al., 1977).

Zeolites are a family of naturally occurring crystalline aluminosilicate minerals that are composed of a 3-D skeleton of symmetrically stacked aluminium (AlO₄) and silica oxides (SiO₄) joined by oxygen atoms (Rozic et al., 2000) (Fig 6.1). The negative charge of the alumina units are normally balanced by the presence of exchangeable cations – notably calcium, magnesium, sodium, potassium and iron, but these are preferably displaced by other substances such as heavy metals and most importantly by ammonium ions (Burgees et al., 2003).

One type of naturally occurring zeolite which has been shown to have a particularly high cation exchange capacity is clinoptilolite, which was formed by the devitrification of volcanic ash in lakes and marine waters millions of years ago. Clinoptilolite is one of the most researched of all the zeolites and is widely regarded as the most useful having been used for decades to reduce the concentration of ammonium in effluents (Mercer et al., 1970) and ammonia in freshwater sediments (Besser et al., 1998). In addition to waste
water and effluent treatment, the unique absorbance qualities of clinoptilolite has meant that is also has a wide range of industry applications including as a plant growth medium, the manufacture of slow release fertilizers, the handling and disposal of nuclear waste, swimming pool and aquarium filtration and as an animal feed additive to reduce the risk of acidosis and ammonia toxicity (www.zeolite.co.nz ; www.zeolife.co.uk).

Fig 5.1 Crystal structure of clinoptilolite

The optimal ammonium adsorption capacity of zeolite (clinoptilolite) has been shown to vary from 4.5mg NH₃-N/g when used to filter sewage (Hlavay, et al., 1982; Booker et al., 1996) to 8.1 – 15.2 NH₃-N/g when used to filter standard solutions (Bernal and Lopez-Real, 1993). Although it has been shown to reduce the ammonium concentration of filtrate to below 1 mg NH₃-N/l (Booker et al., 1996), the ability of zeolite to absorb ammonium ions is dependent on the concentration of zeolite in the host rock, the porosity and density of the host rock, chemical pre-treatment, pH and the presence of competing cations (Booker et al., 1996). Because ammonium needs to diffuse through the zeolite particle before it reaches the site of adsorption, the absorption capacity of zeolite in commercial applications is also dependent on the rate of diffusion and absorption, of which particle size is a significant factor (Booker et al., 1996):

\[ \text{Rate of absorption} \propto \frac{1}{r^2} \]
Absorption of ammonium by zeolite occurs rapidly within the first 10 minutes of contact with the highest rate and level of absorption associated with smaller zeolite particle sizes due to the higher surface area and rate of absorption (Booker et al., 1996). However, when zeolite is employed in filtration systems the level of ammonium absorption is also highly dependent on the flow rate through the column. It has been suggested by Booker et al., (1996) that the bed volume flow rate (BV/h) must be less than 10 BV/h because this enables the system to absorb significantly more ammonia at higher solution concentrations. The optimum flow rate for filtration systems has been suggested to be 5-7 BV/h (Hlavay, et al., 1982; Booker et al., 1996).

The aims of this study were to 1) assess the ammonium absorbance capacity of zeolite (clintonlofilolite) with a diameter of 2-3mm, 2) determine the optimum BV/h and 3) to assess the impact of zeolite (clintonoifilolite) filtration on the microbial population and activity of rumen fluid.
5.3 MATERIAL AND METHODS

5.3.1 EXPERIMENTAL TREATMENTS

EXPERIMENT 1

This experiment was designed to study the effect of bed volume flow rate (BV/h) on the nitrogen absorption coefficient of zeolite when used to filter a standard ammonium solution (2400 mgN/L)

EXPERIMENT 2

This experiment was designed to assess whether filtering rumen liquor through zeolite to remove background nitrogen had any effect on the size or activity of the microbial population during IVGPT.

5.3.2 PREPARATION OF ZEOLITE

The zeolite (Clinoptilolite) was purchased from Euremica Environmental Ltd and had a particle size of 2-3 mm. Because the zeolite was received in its raw state it was first processed and washed to remove any small particles and organic material that could contaminate the filtrate (see Appendix A.1.5)

5.3.3 PREPARATION OF THE STANDARD SOLUTION

A 2400 mgN/l standard solution (Ni) was made by dissolving 67.7g of NH₄HCO₃ in five litres of distilled water.

5.3.4 MEASURING THE NITROGEN ABSORPTION COEFFICIENT OF ZEOLITE AT DIFFERENT BED VOLUME FLOW RATES

Approximately 200g (W₁) of washed and dried zeolite was poured into two glass columns (length 300mm, radius 17.5mm) fitted with a section of rubber tubing and a variable plastic clamp. The glass columns were held in a vertical position under 500ml aspirator bottles connected to the columns with rubber tubing and through a rubber stopper. The columns were fed into 500ml bottles placed underneath through rubber tubing fitted with variable plastic clamps.
With the lower clamp fully open the zeolite was washed three times with distilled water to remove any fine particles and to saturate the zeolite which is highly absorbent. The effect of each washing and the clarity of the water collected was assessed by placing the beaker over a black cross drawn on a white background. Only when the cross was clearly visible was washing stopped.

After the zeolite was washed, the lower clamp was closed and bed volume estimated by pouring measured quantities of distilled water into each column until the zeolite was covered ($V_1$). The lower clamp was then opened and any distilled water allowed to drain out, before the clamp was closed again and the glass column connected to the 500ml aspirator bottles above. 500ml of the standard solution ($V_2$) was poured into each 500ml aspirator bottle using a funnel and allowed to drain into each column. When the column was full, the clamp was opened by one notch, the time recorded and the filtrate collected in the 500ml bottles placed below the column. The clock was stopped and the time recorded when no more solution was present in the column.

This procedure was repeated a further seven times, each time with the clamp opened a further notch to increase the bed volume flow rate.
The nitrogen content of the standard solution ($N_i$) and the nitrogen content of the filtrate ($N_2$) was measured using FOSS FIAstar 5000 Analyzer fitted with a 5027 sampler unit.

The calculations were as follows:

Bed Volume Flow Rate (BV/h) = \((V_2 / V_1) \times (60 / \text{Total minutes})\)

Filter time (litre/h) = \(1000 / (V_1 \times \text{BV/h})\)

Absorption coefficient (mgN/mgzeolite) = \((N_i - N_2) / W_i\)
5.3.5 MEASURING THE EFFECT OF FILTRATION ON THE RUMEN FERMENTATION PROFILE OF SUCROSE DURING IVGPT

The equivalent of 100mg of sucrose was weighed in duplicate into 100 ml glass syringes and placed in the fridge until the morning of incubation. Six blank syringes containing no sucrose were also created, three for the filtered and unfiltered rumen liquor respectively.

On the morning of inoculation and prior to collecting the rumen liquor, the syringes containing the samples, along with ‘blanks’ containing no substrate, were placed in a 39°C water bath.

Rumen liquor was collected from sheep kept outside during summer and grazing predominantly perennial ryegrass with no access to supplemental feeds. One sheep was used for each gas production run. On the morning of inoculation, individual sheep were collected from the pasture at approximately 09:30, slaughtered and the rumen and reticulum removed and placed in a thermos box to keep warm whilst travelling back to the laboratory. At the laboratory the rumen contents were strained through two layers of 250 μm netting and three layers of muslin, and then stored in a thermos flask gassed with CO₂.

The microbial activity of the rumen liquor before filtration was assessed by measuring its absorbance at 600 nm (Nagadi et al., 2000). The dilution ratio with anaerobic medium was then calculated to ensure that the inoculum had an absorbance at 600 nm of 0.111 - 0.134.

Approximately 1 litre of rumen liquor was filtered through 400g of zeolite at a flow rate of 8.92 BV/h (see Appendix A.1.5). The microbial activity of the rumen liquor after filtration was the assessed by measuring its absorbance at 600nm (Nagadi et al., 2000).
Fig I.5.2 Column filtration of rumen liquor through zeolite

The appropriate volume of anaerobic buffer medium (ABM) (Menke and Steingass, 1988) was prepared for both the unfiltered and filtered rumen liquor in two 2l round bottom flasks. These were placed in a 39°C water bath, saturated with CO₂ and sealed with sponge and cling film. When the medium reached 39°C, each rumen liquor was added to the appropriate ABM and the inoculum was gassed with CO₂ for a further 5 minutes. Using an automatic dispensing pump, 30 ml of inoculum was added to each fermentation syringe before it was returned to the water bath. Measurement of in vitro gas production were performed as described by Jessop and Herrero (1996), with cumulative gas production in each syringe read every half hour for the first 3 hours, then hourly until 6 hours and thereafter at 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 56, 64, and 72h. Syringes were manually shaken at each reading.

5.3.6 INTERPRETATION OF GAS PRODUCTION DATA

The fermentation profile of each silage and/or particle size was determined by applying the analytical procedure described in Appendix A1.4.

This initially describe the gas profile of each silage in terms of the volume (A) and rate of gas (Cₐ) produced in the early stages incubation and then after a lag, the volume (B) and rate of gas (Cₐ) produced in the later stages of incubation.
5.3.7 STATISTICAL ANALYSIS

Using the MINITAB statistical package (2000), paired T-tests were used to compare the nitrogen absorbance of zeolite at different bed volume flow rates and the absorbance and cumulative gas production parameters of both the filtered and unfiltered rumen liquor. Analysis of variance (ANOVA) using a general linear model (GLM) was used to compare the nitrogen absorbance of zeolite at different bed volume flow rates.

A three-phase hyperbolic decay curve was used to describe the relationship between the bed volume flow rate and nitrogen absorbance coefficient of zeolite and to generate the prediction equation.
5.4 RESULTS

5.4.1 EXPERIMENT 1 – EFFECT OF BED VOLUME FLOW RATE ON THE AMMONIA ABSORPTION CAPACITY OF ZEOLITE

The effect of bed volume flow rate (BV/h) on the level of nitrogen absorbed by zeolite from a standard solution (2400 mgN/L) is presented in Table 5.1 and illustrated in Fig 5.1.

There was a significant decrease in the quantity of nitrogen absorbed by the zeolite as the BV/h increased (P<0.001) (Table 5.1), but there was no significant difference in the quantity of nitrogen absorbed at filtration rates greater than 68.2 BV/h (P>0.05).

The relationship between BV/h and the absorption coefficient of zeolite was described using a 3-phase hyperbolic decay curve (Fig 5.1) and the equation which describes this curve can be used to predict the absorption coefficient of zeolite at different applied filtration rates (R²=0.998, P<0.001) (Table 5.2).

Table 5.1 Removal of ammonia during filtration of a standard solution (2400mgN/L) through 200g of zeolite at different bed volume flow rates. Means with different superscripts within the same column are significantly different (P<0.05).

<table>
<thead>
<tr>
<th>BV/h</th>
<th>Bed Volume (ml)</th>
<th>Filter Time (hours/litre)</th>
<th>Absorbed N (mgN)</th>
<th>Absorption Coefficient (mgN / g zeolite)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.26</td>
<td>58</td>
<td>3.28</td>
<td>1496.9⁺ (15.7)</td>
<td>3.74⁺ (0.04)</td>
</tr>
<tr>
<td>8.92</td>
<td>58</td>
<td>1.93</td>
<td>1181.3⁺ (38.9)</td>
<td>2.95⁺ (0.09)</td>
</tr>
<tr>
<td>10.34</td>
<td>60</td>
<td>1.61</td>
<td>1165.6⁺ (6.3)</td>
<td>2.91⁺ (0.02)</td>
</tr>
<tr>
<td>26.2</td>
<td>60</td>
<td>0.64</td>
<td>828.3⁺ (46.8)</td>
<td>2.07⁺ (0.12)</td>
</tr>
<tr>
<td>34.53</td>
<td>59</td>
<td>0.49</td>
<td>655.0¹ (31.2)</td>
<td>1.64¹ (0.08)</td>
</tr>
<tr>
<td>47.02</td>
<td>59</td>
<td>0.36</td>
<td>548.8² (17.0)</td>
<td>1.37² (0.04)</td>
</tr>
<tr>
<td>68.19</td>
<td>58</td>
<td>0.25</td>
<td>420.0¹ (26.3)</td>
<td>1.05¹ (0.07)</td>
</tr>
<tr>
<td>87.98</td>
<td>59</td>
<td>0.19</td>
<td>440.8¹ (19.3)</td>
<td>1.10¹ (0.05)</td>
</tr>
</tbody>
</table>

Sig. ***

NS P>0.05  *P<0.05  **P<0.01  ***P<0.001
Fig 5.1 Relationship between base volume flow rate (BV/h) and the absorption of ammonia during filtration of a standard solution (2400mgN/L) through a 220g zeolite column.

Table 5.2 Predicting the absorption coefficient of zeolite at different base volume flow rates (BV/h) using a 3-Phase hyperbolic decay curve

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Equation</th>
<th>R²</th>
<th>SEE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>mgN/g zeolite</td>
<td>$0.5094 + \left(\frac{4.6318 \times 11.2636}{11.2636 + (BV/h)}\right)$</td>
<td>0.989</td>
<td>0.121</td>
<td>***</td>
</tr>
</tbody>
</table>

NS P>0.05 *P<0.05 **P<0.01 ***P<0.001

5.4.2 EXPERIMENT 2 – EFFECT OF FILTRATION OF RUMEN LIQUOR THROUGH ZEOLITE ON MICROBIAL ACTIVITY DURING IVGPT

The effect of filtration on the gas production profile of sucrose is presented in Table 5.3 and illustrated in Fig 5.2.

Filtering rumen liquor through zeolite had no significant effect on its absorbance at 600nm (P>0.05) and therefore had no effect on the microbial concentration of the filtered rumen liquor (P>0.05) (Table 5.3).

Sucrose incubated in filtered rumen liquor produced 2.9% more gas in the first 6 hours (P<0.05), a shorter lag (P<0.001), 7.9% more gas attributed to the slow B fraction (P<0.001) and 1.7% more total gas (P<0.01) than when incubated in unfiltered rumen liquor. There was no significant difference
between filtered and unfiltered rumen liquor with regard to the volume of quick gas attributed to the A fraction (P>0.05), the rate of production of slow gas (B fraction) (P>0.05) or the volume of gas produced after 12 and 24 hours of incubation (P>0.05)

Table 5.3 Estimated microbial concentration and gas production parameters of 220mgDM of sucrose when incubated in ovine rumen liquor before and after filtration through zeolite.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Unfiltered</th>
<th>Filtered</th>
<th>Sig.</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Absorbance @ 600nm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacterial DM (mg/10ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absorbance</td>
<td>4</td>
<td>0.178 (0.052)</td>
<td>0.152 (0.035)</td>
<td>NS</td>
</tr>
<tr>
<td>Bacterial DM</td>
<td>4</td>
<td>153.8 (44.9)</td>
<td>131.7 (29.9)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>A (ml)</strong></td>
<td>12</td>
<td>40.96 (3.03)</td>
<td>40.61 (2.61)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>C&lt;sub&gt;a&lt;/sub&gt; (/h)</strong></td>
<td>12</td>
<td>0.310 (0.008)</td>
<td>0.347 (0.012)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>B (ml)</strong></td>
<td>12</td>
<td>22.77 (0.811)</td>
<td>24.56 (0.92)</td>
<td>***</td>
</tr>
<tr>
<td><strong>C&lt;sub&gt;b&lt;/sub&gt; (/h)</strong></td>
<td>12</td>
<td>0.052 (0.004)</td>
<td>0.047 (0.003)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Lag</strong></td>
<td>12</td>
<td>6.38 (0.21)</td>
<td>6.14 (0.19)</td>
<td>***</td>
</tr>
<tr>
<td><strong>Gas @ 6 hrs</strong></td>
<td>12</td>
<td>34.59 (2.55)</td>
<td>35.58 (2.53)</td>
<td>*</td>
</tr>
<tr>
<td><strong>Gas @12 hrs</strong></td>
<td>12</td>
<td>45.74 (2.65)</td>
<td>45.92 (3.06)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Gas @ 24 hrs</strong></td>
<td>12</td>
<td>54.60 (2.74)</td>
<td>54.60 (3.58)</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Total gas (ml)</strong></td>
<td>12</td>
<td>62.97 (3.07)</td>
<td>64.07 (3.54)</td>
<td>**</td>
</tr>
</tbody>
</table>

The cumulative gas production curves were fitted to the equation: \( \text{Gas} = A(1-\exp(-cA/t))+B(1-\exp(-cB(t-Lag)))*\exp(5(t-Lag))/(1+\exp(5(t-Lag))) \) modified from Jessop and Herrero (1996). [see text for interpretation of parameters]. Standard deviation values are shown in brackets.

NS P>0.05 *P<0.05 **P<0.01 ***P<0.001
Fig 5.2 Mean cumulative gas production of 220mgDM of sucrose (n=6) incubated in ovine rumen liquor before (black square) and after (white triangle) filtration through zeolite. Cumulative gas production curves were fitted to the equation: \( \text{Gas} = A(1-\exp(-cA t)) + B(1-\exp(-cB(t-Lag))) \exp(5(t-Lag))/\left(1+\exp(5(t-Lag))\right) \) modified from Jessop and Herrero (1996) [see text for interpretation of parameters].
5.5 DISCUSSION

5.5.1 EXPERIMENT 1 – EFFECT OF BED VOLUME FLOW RATE ON THE AMMONIA ABSORPTION CAPACITY OF ZEOLITE

The efficiency of commercial filtration systems is dependent not only on the absorbance capacity of the active material but also its rate of absorbance as it is this that determines the most economical filtration rate for the system (Booker et al., 1996). This was illustrated in this study where an increase in the bed volume flow rate (BV/h) was related to a significant decrease in the absorbance capacity of zeolite during column filtration of an ammonium solution (P<0.001), but only up to a flow rate of 68.2 BV/h above which there was no further decrease in absorption coefficient (P>0.05) (Table 5.1). This indicates that zeolite has a minimum absorption coefficient of ~1mgNH₃-N/g zeolite and may have possible applications in rapid filtration systems requiring low levels of ammonium absorption.

The effect of the flow rate on the absorption coefficient of ammonium by zeolite can be predicted using a 3-phase hyperbolic decay curve (Fig 5.1) (R²=0.998, P<0.001) (Table 5.2) allowing end-users to adapt the efficacy of the technique to their own requirements.

The results of this study suggest that the optimum flow rate for filtration through 2-3mm zeolite is 8.93 BV/h, which is within the range of target flow rates suggested by Booker et al., (1996) but above the optimal flow rate of 5-7 BV/h suggested by Hlavay, et al., (1982) and Booker et al., (1996). A flow rate of 5.26 BV/h is achievable using this zeolite system but it requires a significantly longer filtration time compared to 8.93 BV/h (P<0.05) (Table 5.1). This may have implications with regard to microbial cooling if zeolite is used to remove ammonia from rumen liquor.

Because a flow rate of 8.93 BV/h equates to an absorption coefficient of 2.95mgNH₃-N/g zeolite (Table 5.1), filtering 1L of rumen liquor through 400g of zeolite equates to an absorption potential of 1180 mgNH₃-N/l, which is much higher than any expected rumen ammonia level.
5.5.2 EXPERIMENT 2 – EFFECT OF FILTRATION OF RUMEN LIQUOR THROUGH ZEOLITE ON MICROBIAL ACTIVITY DURING IVGPT

The results showed that filtration of rumen liquor had no significant affect on the size of the microbial population (P>0.05), indicating that rumen bacteria are not selectively retained in the zeolite column (Table 5.3).

Using filtered rumen liquor to incubate sucrose also showed that filtration had no detrimental effect on microbial activity during IVGPT (P>0.05) (Table 5.3). This is in agreement with Burges et al., (2003) who showed no toxic effects of zeolite on amphipod (Ampelisca abdita) and mysid (Americanmysis bohia) organisms. In contrast, the results of this study indicate that microbial activity was significantly improved following filtration. Filtered rumen liquor produced 2.9% more gas in the first 6 hours (P<0.05), had a shorter lag (P<0.001), produced 7.9% more slow gas (B fraction) (P<0.001) and 1.7% more total gas (P<0.01) compared to unfiltered rumen liquor.

Whether such an increase in gas production would still be statistically significant if consideration was given to the variation in microbial activity between donor animals (Nagadi, 1999; Nagadi et al., 2000) is unknown. However, although there was no significant decrease in the estimated microbial population after filtration there was a slight decrease in its absorption at 600nm. This suggests that filtration may remove a component of rumen liquor that would normally have a negative effect on microbial performance and this may be related to retention of protozoa in the zeolite. These protozoa commonly predate on rumen bacteria but are also considerably larger (1-1000μm vs. 0.64-1.5μm) and therefore have a greater chance of being retained in the zeolite. If this were true the improvement in microbial performance would be in agreement with other authors who have shown that in vivo defaunation results in an increase in microbial growth efficiency (Eugène et al., 2004), animal performance and feed conversion efficiency (Santra and Karim, 2000; Eugène et al., 2004).
5.6 CONCLUSION

The absorption coefficient of zeolite significantly decreases as the bed volume flow rate through the column increases. The absorption coefficient of zeolite can be predicted from the specific bed volume flow rate using a 3-phase hyperbolic decay curve ($R^2=0.998$, $P<0.001$).

The optimum practical flow rate for filtration of rumen liquor is 8.93 BV/h which equates to an absorption coefficient of 2.95mgNH$_3$N/g zeolite.

Filtration of rumen liquor through zeolite has no effect on the size of the bacterial population but it improves microbial performance during IVGPT. This could possibly be related to retention of protozoa in zeolite which would otherwise predate on rumen microbes and reduce the effective microbial population.
CHAPTER 6

Comparison of the protein degradation parameters of grass silage estimated using the nylon bag technique and IVGPT
Degradation of feed protein in the rumen is commonly assessed using an in vivo nylon bag technique (Ørskov and McDonald, 1979), which is not only tedious and expensive but also has a poor repeatability. The purpose of this study was to compare the protein degradation parameters of grass silage estimated using the in vivo nylon bag technique and a modified in vitro gas production technique (IVGPT)

The nylon bag technique overestimated the concentration of soluble nitrogen in grass silage by 23% and the estimated protein A fraction was significantly higher than that estimated using IVGPT (P<0.001). Using IVGPT, the estimated protein A fraction was correlated to the concentration of soluble non-ammonia nitrogen (SNAN) (R²=77.9, P<0.001) with the results indicating that only 76.3% of grass silage SNAN is quickly degraded with up to 23.7% being slowly degradable.

Using the modified IVGPT technique, the estimated concentration of quickly degradable protein (QDP) was 52.7% lower (P<0.001) than that estimated using the nylon bag technique, which also estimated a significantly higher concentration of slowly degradable protein (SDP) (P<0.001). The estimated concentration of effective rumen degradable protein (ERDP) was 16.7% less using IVGPT but the estimated concentration of digestible undegraded protein (DUP) was 155.7% higher (P<0.001) than that estimated using the nylon bag technique.

These results indicate that the modified IVGPT technique may provide a clearer understanding of feed protein degradation in the rumen because not all material lost from the nylon bag was shown to be soluble or quickly degraded.
6.2 INTRODUCTION

The most expensive component of ruminant diets is the supplementation of dietary protein to meet the protein demands of the animal. Consequently, a primary aim of diet formulation is to maximise microbial growth, which not only increases the efficiency of diet utilisation but also increases the supply of microbial protein to the small intestine, therefore decreasing the requirement for expensive supplementation with feeds high in digestible undegraded protein (DUP) (Leng, 1993; Blummel et al., 1999).

Ruminal microbes degrade feed protein using extra cellular proteinases that partially hydrolyse the peptide bonds of the protein chain (Russel et al., 1992) resulting in a mixture of peptides and amino acids. These are transported across the cell membrane and into the microbial cell where the peptides are further hydrolysed into amino acids. Microbes utilise amino acids by either incorporating them into microbial protein or by using them as an energy source and excreting ammonia back into the rumen liquor (Tamminga, 1979). The majority of this excreted ammonia is absorbed across the rumen wall and excreted in the urine, but depending on the availability of quickly fermentable carbohydrate a proportion will be utilised by other microbes for microbial protein synthesis (MPS) (Coleman, 1967; Nolan, 1993).

In addition to those present as a direct result of proteolysis of feed protein, a significant proportion of the amino acids and peptides found in the rumen are microbial in origin. Although living microbes can excrete amino acids and peptides these free amino acids and peptides are primarily present due to proteolytic recycling of lysed cells or excretion from protozoa following ingestion of microbes (Wallace and McPherson, 1987) or fungi (Newbold and Hillman, 1990) and can account for over 50% of the total nitrogen used for MPS (Leng and Nolan, 1984).

Although the majority of ruminal bacteria can survive solely on non-protein nitrogen (NPN), when microbes are utilising rapidly fermenting carbohydrate the efficiency of microbial growth and MPS is enhanced by the addition of amino acids and peptides (Griswold et al., 1996; Russel, 1998). Therefore although there is no beneficial effect on microbes utilising structural carbohydrate, addition of amino nitrogen has a positive effect on the growth of microbes utilising starch, pectin and sugars which have been shown to derive 66% of their nitrogen from peptides and amino acids and 34% from ammonia (Russel et al., 1992). Conversely, MPS is also dependent
on the type of carbohydrate present (Dewhurst et al., 1999) with soluble sugars such as sucrose, lactose and fructose shown to be superior to starch for the fixation of microbial nitrogen in the rumen (Chamberlain et al., 1993).

The influence of nitrogen and carbohydrate availability on MPS is recognized in the UK metabolizable protein (MP) system but there are concerns as to the assumed extent and efficiency of MPS from quickly (QDP) and slowly degradable protein (SDP) (Webster et al., 2003).

When energy availability in the rumen is not limiting, the MP system assumes that microbes can only utilise QDP with an efficiency of 80%, whilst the efficiency of SDP utilisation is assumed to be 100% (AFRC, 1993). It has been suggested by other authors that assuming a lower efficiency of QDP capture by rumen microbes encourages the overfeeding of dietary protein compared to other systems (Sniffen et al., 1992). However, recent in vivo work has demonstrated that not only is there a difference in the efficiency of microbial capture of QDP and SDP but the current assumption may still overestimate the efficiency of QDP capture which has been suggested to be 0.5 (Webster et al., 2003).

The kinetics of protein degradation used in the MP system are determined by incubating feeds in nylon bags that are placed in the rumen or in buffered rumen fluid and measuring the nitrogen content of the feed residues at various time points during the incubation (Ørskov et al., 1980). It is assumed that all nitrogen leaving the nylon bag is soluble and immediately degradable, but fine feed particles can also leave the bag undegraded and may result in the overestimation of protein degradability (Chen et al., 1987). It has also been shown that soluble protein may not be immediately degraded and can remain in the rumen for 8-12 hours (Fahmy et al., 1991; Aufrère et al., 1994). Soluble protein in the liquid phase of rumen passage can therefore make a significant contribution to the by-pass protein fraction of feeds with 11% of non-ammonia nitrogen (NAN) in lupins and 8% of the NAN in peas shown to escape rumen degradation (Aufrère et al., 2001).

In addition to being tedious, the nylon bag technique is also limited by the inaccurate differentiation between undegraded feed proteins and microbial protein (Broderick, 1987). Microbial colonisation of feed occurs in the first 8 hours of incubation and microbial protein can account for up to 72% of the residual protein remaining in the bag after 48 hours (Alexandrov, 1998) and may result in the underestimation of nitrogen degradation (Negi et al., 1988; Becker et al., 1995). In an attempt to remove the interference of microbial
protein residue from the nylon bags, laboratories commonly employ a post incubation wash in cold water, but because there is no standard washing procedure there is a significant error both within and between different laboratories (Tuori et al., 1998).

There is therefore a requirement to develop a relatively simple but reliable laboratory method for estimating the protein degradation of feedstuffs. The in vitro gas protein technique (IVGPT) (Menke and Steingass, 1988), which is widely used to estimate the rumen degradation profile of carbohydrates, is also sensitive to nitrogen availability (Nagadi, 1999) especially in the first 8 hours of incubation when a there is a linear relationship between gas production and MPS (Krishnamorthy et al., 1991). Using IVGPT as an index of MPS, the technique has been successfully used to measure the availability of feed protein to hindgut microbes (Cone et al., 2005) and it is proposed that IVGPT could be applied to assess the kinetics of feed protein degradation in the rumen. The advantage of using IVGPT is that it provides a larger number of data points than is possible using the nylon bag technique and as a consequence may enable the accurate description of QDP fermentation rather than its assumed degradation via loss from a nylon bag.

The development of IVGPT to measure the kinetics of protein degradation is described in detail in previous chapters. These studies indicated that IVGPT was sensitive to nitrogen availability and could be applied to measure the protein degradation of feed in the rumen by incubating a weight of sample equating to 78.4mgN/l in 30ml of buffered nitrogen free rumen liquor with 100mg of sucrose.

The aim of this study was to compare the protein degradation parameters of grass silage estimated using IVGPT with that estimated using in vivo nylon bag measurements.
6.3 MATERIAL AND METHODS

6.3.1 EXPERIMENTAL AIM

The aim of this experiment was to compare the estimated protein degradation parameters of grass silage incubated either in vivo using the nylon bag technique or in vitro using a new IVGPT technique.

6.3.2 PROXIMATE ANALYSIS

Samples of 13 grass silages made from predominantly perennial ryegrass were obtained from commercial farms throughout Northern Ireland by the Agricultural Research Institute of Northern Ireland (ARINI).

All the proximate analysis of the grass silages were completed by ARINI.

The DM content (g/kg) of each forage sample was measured by drying approximately 100g of fresh material in a 60°C oven for 24 hours. The dried samples were then milled through a 1mm sieve using a Retsch© mill, analysed for NDF (g/kgDM) (Van Soest et al., 1991) and ADF (g/kgDM) using ANKOM F57© fibre bags and for crude protein (CP) (g/kgDM) using the standard Kjedahl technique. See Appendix A.1 for details of the methodology used. The lactic acid (g/kgDM), total volatile fatty acid (VFA) (g/kgDM), ammonia (g/kgDM) content and pH of each of the silages was estimated using NIRS (Foss NIRSystem 6500).

Sub-samples of the fresh silages were roughly cut with scissors and also analysed for CP using the standard Kjedahl technique. The soluble nitrogen content (g/kgDM) of the grass silage was determined by placing the fresh silage in F57© fibre bags that have a pore size of 25μm, washing with cold water and then measuring the level of insoluble nitrogen remaining:

\[
\text{Soluble N (g/kgDM)} = \text{Total N (g/kgDM)} - \text{Insoluble N (g/kgDM)}
\]
6.3.3 ESTIMATING THE PROTEIN DEGRADATION PROFILE OF FRESH GRASS SILAGE USING THE IN VIVO NYLON BAG TECHNIQUE

The following work was carried out by ARINI who gave kind permission for the results to be used in this study. The experimental design and methodology used are described by Steen et al., (1998) and Dawson and Steen (2000).

The in vivo study used three Aberdeen x Friesian steers fitted with permanent ruminal cannulae, and offered a basal diet of grass silage ad libitum for a period of at least 3 weeks prior to the experiment. The steers were individually tethered in the stalls and exercised regularly throughout the study.

The rates of disappearance and degradability of total nitrogen were determined using nylon bags as described by Mehrez and Ørskov (1977) and Ørskov et al., (1980). The nylon bags were made from polyester filter cloth (Locker, Wire Weavers Limited) with a pore size of 1600 µm. The bags were double sewn with rounded corners to ensure a smooth interior free of pockets and crevices. Sufficient fresh silage was put in each nylon bag to provide approximately 5 g of DM.

The 13 silages were incubated in the rumen of the three steers in a randomised block design with the 3 steers being the 3 blocks. The experiment consisted of two periods each of two weeks duration. In period one, silages 1 to 8 were incubated in each steer, in triplicate for each time interval. In period two, the next five silages were incubated in each steer. This allowed a total of three observations per silage per incubation time.

The bags were tied with nylon cord and inserted into the rumen of each steer. A total of eight silages were inserted into the rumen of each steer (24 bags per steer) and all were removed after 6 hours. This procedure was repeated with the bags being removed after 12, 24, 48 and 72 hours. One set of bags was also prepared and washed immediately to determine the soluble fraction (time 0 hours). After removal from the rumen the bags were washed and dried at 60°C for 48 hours. The proportion of silage DM degraded after each time interval was calculated for each bag. The residues from each time point were collected from each steer, mixed together and analysed for nitrogen concentration. The proportion of nitrogen degraded at each time interval was calculated for each steer and the results grouped.
The results from nitrogen disappearance were fitted to the exponential equation (Ørskov and McDonald, 1979): \( P = a + b (1 - e^{-ct}) \) where \( P \) is the proportion of total nitrogen degraded at time \( t \), \( a \) is the soluble nitrogen fraction, \( b \) is the slowly degradable fraction and \( c \) is the fractional rate of degradation of \( b \).

The effective protein degradability of each grass silage was calculated assuming an outflow rates (Kp) of 0.08/h (AFRC, 1993):

- **Quickly Degradable Protein (QDP) (%CP)**
  \[ = a (\%CP) \]

- **Slowly Degradable Protein (SDP) (%CP)**
  \[ = (b (\%CP) + c (\%CP) + 0.08)) \]

- **Effective Rumen Degradable Protein (ERDP) (%CP)**
  \[ = (0.8 * QDP (%CP)) + SDP (%CP) \]

- **Digestible Undegraded Protein (DUP) (%CP)**
  \[ = \frac{(0.9 * (1 - QDP(%CP) * CP(\%g/\%kgDM)) - (SDP (%CP) * CP(\%g/\%kgDM)) - 6.25) / CP \%g/\%kgDM)}{CP \%g/\%kgDM} \]

### 6.3.4 PREPARATION OF ZEOLITE

The zeolite (Clinoptilolite) was purchased from Euremica Environmental Ltd and had a particle size of 2-3 mm. Because the zeolite was received in its raw state it was first processed and washed to remove any small particles and organic material that could contaminate the filtrate (see Appendix A.1.5)

### 6.3.5 ESTIMATING THE PROTEIN DEGRADATION PROFILE OF DRIED AND MILLED GRASS SILAGE USING IVGPT

All the 100ml glass syringes were primed with approximately 100mg of sucrose and then a weight of grass silage equating to 78.4 mgN/L was weighed in triplicate into the appropriate glass syringes:

\[ \text{Grass silage (mg)} = \frac{((1000 / \text{CP (g/kgDM)}) * 0.1875 \text{mgCP/30ml}) * 78.4 \text{mgN/L}}{\text{CP g/kgDM}} \]

For each gas run, three additional blank syringes were also prepared containing 100mg of sucrose but no grass silage sample.

On the morning of inoculation and prior to collecting the rumen liquor, the syringes containing the samples, along with 'blanks' containing no substrate, were placed in a 39°C water bath.
Rumen liquor was collected from sheep kept outside during summer and grazing predominantly perennial ryegrass with no access to supplemental feeds. On the morning of inoculation, individual sheep were collected from the pasture at approximately 09:30, slaughtered and the rumen and reticulum removed and placed in a thermos box to keep warm whilst travelling back to the laboratory. At the laboratory the rumen contents were strained through two layers of 250 μm netting and three layers of muslin, and then stored in a thermos flask gassed with CO₂.

The microbial activity of the rumen liquor before filtration was assessed by measuring its absorbance at 600 nm (Nagadi et al., 2000). The dilution ratio with anaerobic medium was then calculated to ensure that the inoculum had an absorbance at 600 nm of 0.111 - 0.134.

Approximately 1 litre of rumen liquor was filtered through 400g of zeolite at a flow rate of 8.92 BV/h (see Appendix A.1.5), stored in a thermos flask and gassed with CO₂.

The appropriate volume of nitrogen free ABM (Menke and Steingass, 1988) was prepared (see Appendix A.1.5), placed in a 39°C water bath and saturated with CO₂. When the medium reached 39°C, the filtered rumen liquor was added and the inoculum was gassed with CO₂ for a further 5 minutes. Using an automatic dispensing pump, 30 ml of inoculum was added to each fermentation syringe before it was returned to the water bath. Measurement of in vitro gas production were performed as described by Jessop and Herrero (1996), with cumulative gas production in each syringe read every half hour for the first 3 hours, then hourly until 6 hours and thereafter at 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 56, 64, and 72h. Syringes were manually shaken at each reading.

After 72h of incubation the residual nitrogen concentration was measured by firstly emptying each syringe into 200ml flat medicinal bottles containing 15ml of NDF solution and autoclaving at 105°C for 1 hour to solubilise microbial protein. The contents of each bottle were filtered through Whatman 54 Hardened filter paper and the residual nitrogen measured using the standard Kjeldhal technique (see Appendix A.1.5).
6.3.6 INTERPRETATION OF GAS PRODUCTION DATA

The protein degradation profile of each grass silage was determined by applying the analytical procedure described in Appendix A1.5. This initially describes the protein degradation of grass silage in terms of the proportion (A) and rate (C_3) of quickly fermentable protein and then after a lag, the proportion (B) and rate (C_3) of slowly fermentable protein (SCP). Assuming an outflow rate (K_p) of 0.08/h (AFRC, 1993) these degradation parameters are then used to estimate the effective protein degradability of each grass silage in terms of the proportion of quickly (QDP) and slowly (SDP) degradable protein, the effective rumen degradable protein (ERDP) and digestible undegraded protein (DUP).

6.3.7 STATISTICAL ANALYSIS

Using the MINITAB statistical package (2000), paired T-tests were used to compare the protein degradation parameters of the grass silage estimated using the in vivo nylon bag or IVGPT techniques.

Linear regression was used to assess the relationship between soluble nitrogen and the QDP fraction estimated by the two techniques. Outliers were removed using the ‘unusual observation function’ within the Minitab (2000), which removes observations with a standardized residual greater than 2.
6.4 RESULTS

6.4.1 PROXIMATE ANALYSIS

The proximate analyses results for the forage samples studied are presented in Table 6.1.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM (g/kg)</td>
<td>244.3</td>
<td>174.9</td>
<td>413.3</td>
<td>77.1</td>
</tr>
<tr>
<td>NDF (g/kgDM)</td>
<td>528.2</td>
<td>413.3</td>
<td>619.3</td>
<td>60.8</td>
</tr>
<tr>
<td>ADF (g/kgDM)</td>
<td>338.2</td>
<td>277.18</td>
<td>391.03</td>
<td>33.34</td>
</tr>
<tr>
<td>CP (g/kgDM)</td>
<td>137.42</td>
<td>89.72</td>
<td>171.19</td>
<td>25.57</td>
</tr>
<tr>
<td>Soluble N (g/kgDM)</td>
<td>80.34</td>
<td>46.34</td>
<td>118.47</td>
<td>23.28</td>
</tr>
<tr>
<td>Ammonia N (g/kgDM)</td>
<td>0.534</td>
<td>0.316</td>
<td>0.916</td>
<td>0.203</td>
</tr>
</tbody>
</table>

6.4.2 COMPARISON OF THE PROTEIN DEGRADATION PROFILE OF GRASS SILAGE ESTIMATED USING THE IN VIVO NYLON BAG TECHNIQUE AND IVGPT

The estimated protein degradation parameters of fresh grass silage incubated in vivo in nylon bags are presented in Table 6.2, whilst the protein degradation parameters of dried grass silage estimated using IVGPT are presented in Table 6.3 and illustrated in Fig 6.1. A comparison of the protein degradation profile estimated by both techniques is presented in Table 6.4 and illustrated in Fig. 6.2.

As a proportion of the soluble nitrogen concentration, the estimated protein A fraction was 38.1% lower using IVGPT compared to the nylon bag technique (Table 6.4), which estimated the protein A fraction to be 23% higher than the soluble nitrogen concentration (Table 6.2). In contrast, using IVGPT it was estimated that only 78.3% of soluble non-ammonia nitrogen (NAN) is quickly fermented and up to 37.5% may be slowly fermented (Table 6.3).

Although there was no significant difference in the estimated rate of degradation of the protein B fraction (C₅) or the concentration of undegraded protein (U) (P>0.05) between the two analytical techniques, the protein A
fraction was 40.2% lower (P<0.001) during IVGPT which also estimated the protein B fraction to be 122% higher (P<0.001) than that estimated using the nylon bag technique (Table 6.4).

Consequently, the estimated proportion of QDP is 52% lower (P<0.001) using IVGPT which also estimates the proportion of SDP to be 104.5% higher (P<0.001) than that estimated using the nylon bag technique. The final estimates of ERDP and DUP were 16.7% lower (P<0.001) and 155.7% higher (P<0.001) respectively using IVGPT compared to the nylon bag technique.

Table 6.2 Estimated protein degradation parameters of fresh grass silage using the in-vivo nylon bag technique (n=13). The samples were cut with scissors and incubated in triplicate.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (prop CP)</td>
<td>0.687</td>
<td>0.609</td>
<td>0.780</td>
<td>0.054</td>
</tr>
<tr>
<td>B (prop CP)</td>
<td>0.228</td>
<td>0.116</td>
<td>0.313</td>
<td>0.074</td>
</tr>
<tr>
<td>C (/h)</td>
<td>0.078</td>
<td>0.058</td>
<td>0.107</td>
<td>0.016</td>
</tr>
<tr>
<td>U (prop CP)</td>
<td>0.086</td>
<td>0.038</td>
<td>0.171</td>
<td>0.041</td>
</tr>
<tr>
<td>A (% sol N)</td>
<td>1.233</td>
<td>0.950</td>
<td>1.574</td>
<td>0.186</td>
</tr>
<tr>
<td>QDP @ 0.08/h (prop CP)</td>
<td>0.687</td>
<td>0.609</td>
<td>0.779</td>
<td>0.054</td>
</tr>
<tr>
<td>SDP @ 0.08/h (prop CP)</td>
<td>0.110</td>
<td>0.057</td>
<td>0.158</td>
<td>0.035</td>
</tr>
<tr>
<td>ERDP @ 0.08/h (prop CP)</td>
<td>0.659</td>
<td>0.605</td>
<td>0.691</td>
<td>0.026</td>
</tr>
<tr>
<td>DUP @ 0.08/h (prop CP)</td>
<td>0.140</td>
<td>0.103</td>
<td>0.188</td>
<td>0.027</td>
</tr>
</tbody>
</table>

Table 6.3 Estimated protein degradation parameters of dried grass silage using IVGPT (n=13). The samples were dried at 60°C for 24h, milled through a 1mm screen and incubated in triplicate.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (prop CP)</td>
<td>0.411</td>
<td>0.273</td>
<td>0.526</td>
<td>0.064</td>
</tr>
<tr>
<td>Ca (/h)</td>
<td>0.3000</td>
<td>0.3000</td>
<td>0.3000</td>
<td>0.000</td>
</tr>
<tr>
<td>B (prop CP)</td>
<td>0.504</td>
<td>0.415</td>
<td>0.649</td>
<td>0.066</td>
</tr>
<tr>
<td>Cb (/h)</td>
<td>0.0791</td>
<td>0.0617</td>
<td>0.096</td>
<td>0.0112</td>
</tr>
<tr>
<td>Lag (h)</td>
<td>1.247</td>
<td>1.092</td>
<td>1.385</td>
<td>0.1035</td>
</tr>
<tr>
<td>U (prop CP)</td>
<td>0.086</td>
<td>0.032</td>
<td>0.156</td>
<td>0.034</td>
</tr>
<tr>
<td>A (% SNAN)</td>
<td>0.763</td>
<td>0.625</td>
<td>0.885</td>
<td>0.084</td>
</tr>
<tr>
<td>QDP @ 0.08/h (prop CP)</td>
<td>0.324</td>
<td>0.215</td>
<td>0.416</td>
<td>0.051</td>
</tr>
<tr>
<td>SDP @ 0.08/h (prop CP)</td>
<td>0.225</td>
<td>0.161</td>
<td>0.313</td>
<td>0.042</td>
</tr>
<tr>
<td>ERDP @ 0.08/h (prop CP)</td>
<td>0.549</td>
<td>0.499</td>
<td>0.615</td>
<td>0.032</td>
</tr>
<tr>
<td>DUP @ 0.08/h (prop CP)</td>
<td>0.358</td>
<td>0.310</td>
<td>0.399</td>
<td>0.022</td>
</tr>
</tbody>
</table>
Fig 6.1 Estimated mean protein degradation profile of dried and milled grass silage (n=13) incubated in triplicate using IVGPT. Totally degradable protein (black circle), quickly degradable protein (QDP) (white square) and slowly degradable protein (SDP) (black triangle).

Table 6.4 Comparison of the protein degradation parameters of grass silage (n=13) estimated from fresh samples incubated in the in vivo using nylon bag method and from dried samples using IVGPT. All samples were incubated in triplicate (n=3). Dried samples were dried at 60°C for 24h and milled through a 1mm screen. Fresh samples were cut with scissors prior to incubation. Standard deviation values are shown in brackets.

<table>
<thead>
<tr>
<th>Type</th>
<th>In vivo nylon bag</th>
<th>In vitro IVGPT</th>
<th>Sig.</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A (prop CP)</td>
<td>0.687 (0.054)</td>
<td>0.411 (0.064)</td>
<td>***</td>
<td>-40.2</td>
</tr>
<tr>
<td>Ca (l/h)</td>
<td>-</td>
<td>0.3000 (0.000)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B (prop CP)</td>
<td>0.227 (0.074)</td>
<td>0.504 (0.066)</td>
<td>***</td>
<td>+122.0</td>
</tr>
<tr>
<td>Cb (l/h)</td>
<td>0.0781 (0.0162)</td>
<td>0.0791 (0.0112)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Lag (h)</td>
<td>-</td>
<td>1.247 (0.1035)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>U(prop CP)</td>
<td>0.086 (0.041)</td>
<td>0.086 (0.034)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>A (% sol N) or (% SNAN)</td>
<td>1.233 (0.186)</td>
<td>0.763 (0.084)</td>
<td>***</td>
<td>-38.1</td>
</tr>
<tr>
<td>QDP @ 0.08/h (prop CP)</td>
<td>0.687 (0.054)</td>
<td>0.324 (0.051)</td>
<td>***</td>
<td>-52.8</td>
</tr>
<tr>
<td>SDP @ 0.08/h (prop CP)</td>
<td>0.110 (0.035)</td>
<td>0.225 (0.042)</td>
<td>***</td>
<td>+104.5</td>
</tr>
<tr>
<td>ERDP @ 0.08/h (prop CP)</td>
<td>0.659 (0.026)</td>
<td>0.549 (0.032)</td>
<td>***</td>
<td>-16.7</td>
</tr>
<tr>
<td>DUP @ 0.08/h (prop CP)</td>
<td>0.140 (0.027)</td>
<td>0.358 (0.022)</td>
<td>***</td>
<td>+155.7</td>
</tr>
</tbody>
</table>

NS P>0.05 *P<0.05 **P<0.01 ***P<0.001
Fig 6.2 Comparison of the estimated protein degradation profiles of dried and milled grass silage \((n=13)\) incubated using IVGPT (black circle) and the same grass silage \((n=13)\) incubated in its fresh state using the in vivo nylon bag technique (white triangle). All samples were incubated in triplicate.

6.4.3 RELATIONSHIP BETWEEN SOLUBLE NITROGEN AND THE PROTEIN ‘A’ FRACTION ESTIMATED BY THE NYLON BAG TECHNIQUE AND IVGPT

The relationships between the soluble nitrogen concentration of grass silage and the protein A fraction estimated using IVGPT and the in vivo nylon bag technique are presented in Table 6.5 and illustrated in Fig 6.3.

There was no significant linear relationship between the soluble nitrogen concentration of grass silage and the protein A fraction estimated using the nylon bag technique \((P>0.05)\), but there was a significant positive linear relationship with the protein A fraction estimated using IVPGT \((R^2=77.9, P<0.001)\) (Table 6.5).

Table 6.5 Linear relationship between the soluble nitrogen content (prop CP) of grass silage \((n=13)\) and the protein ‘A’ fraction (prop CP) estimated from dried and milled samples using IVGPT (black circle) and from fresh samples using the in vivo nylon bag technique (white triangle). All samples were incubated in triplicate.

<table>
<thead>
<tr>
<th>Method Type</th>
<th>(n)</th>
<th>Equation</th>
<th>(R^2)</th>
<th>SEE</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVGPT</td>
<td>12</td>
<td>(0.164 + 0.851 \times A) (prop CP)</td>
<td>77.9</td>
<td>0.038</td>
<td>***</td>
</tr>
<tr>
<td>Nylon Bag</td>
<td>12</td>
<td>(0.438 + 0.193 \times A) (prop CP)</td>
<td>1.6</td>
<td>0.082</td>
<td>NS</td>
</tr>
</tbody>
</table>

\(NS\) \(P>0.05\) \(*P<0.05\) \(**P<0.01\) \(**P<0.001\)
Fig 6.3 Comparison between the soluble nitrogen content of grass silage (n=13) and the protein ‘a’ fraction estimated from dried and milled samples using IVGPT (black circle) and from fresh samples using the in vivo nylon bag technique (white triangle). All samples were incubated in triplicate.

6.4.4 REPEATABILITY OF IVGPT IN ESTIMATING THE PROTEIN DEGRADATION PARAMETERS OF GRASS SILAGE

The protein degradation parameters of a standard grass silage estimated using IVGPT is presented in Table 6.3.

Assuming a T-value larger than 10 is an indicator of good repeatability, IVGPT was shown to have a good repeatability for all protein degradation parameters with the exception of the rate of degradation of the protein B fraction and the concentration of undegraded protein, which had T-values below 10.

Table 6.6 Estimated protein degradation parameters for one dried and milled grass silage sample incubated 10 times (n=10) in IVGPT.

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>Min</th>
<th>Max</th>
<th>SD</th>
<th>T</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (prop CP)</td>
<td>0.461</td>
<td>0.424</td>
<td>0.523</td>
<td>0.035</td>
<td>15.3</td>
</tr>
<tr>
<td>Ca (/h)</td>
<td>0.306</td>
<td>0.300</td>
<td>0.316</td>
<td>0.005</td>
<td>-</td>
</tr>
<tr>
<td>B (prop CP)</td>
<td>0.499</td>
<td>0.432</td>
<td>0.561</td>
<td>0.035</td>
<td>12.3</td>
</tr>
<tr>
<td>Cb (/h)</td>
<td>0.078</td>
<td>0.068</td>
<td>0.092</td>
<td>0.009</td>
<td>8.67</td>
</tr>
<tr>
<td>Lag (hours)</td>
<td>1.119</td>
<td>1.051</td>
<td>1.254</td>
<td>0.066</td>
<td>16.9</td>
</tr>
<tr>
<td>U (prop CP)</td>
<td>0.039</td>
<td>0.015</td>
<td>0.045</td>
<td>0.013</td>
<td>3</td>
</tr>
<tr>
<td>QDP @ 0.08/h (prop CP)</td>
<td>0.366</td>
<td>0.337</td>
<td>0.413</td>
<td>0.027</td>
<td>13.6</td>
</tr>
<tr>
<td>SDP @ 0.08/h (prop CP)</td>
<td>0.225</td>
<td>0.186</td>
<td>0.265</td>
<td>0.026</td>
<td>8.65</td>
</tr>
<tr>
<td>ERDP @ 0.08/h (prop CP)</td>
<td>0.591</td>
<td>0.566</td>
<td>0.608</td>
<td>0.013</td>
<td>45.5</td>
</tr>
<tr>
<td>DUP @ 0.08/h (prop CP)</td>
<td>0.328</td>
<td>0.312</td>
<td>0.350</td>
<td>0.012</td>
<td>27.3</td>
</tr>
</tbody>
</table>
6.5 DISCUSSION

6.5.1 COMPARISON OF THE PROTEIN DEGRADATION PARAMETERS OF GRASS SILAGE ESTIMATED USING THE NYLON BAG TECHNIQUE AND IVGPT

The estimation of feed protein degradation characteristics using the nylon bag technique is limited by the assumption that only soluble feed protein is quickly degraded and at an infinite rate. However, because the pore size of the nylon bags used in this technique is relatively large (1600µm), a proportion of this water-soluble fraction may be composed of insoluble fine feed particles. This study showed that the concentration of soluble nitrogen estimated using the nylon bag technique was 23% higher than the standard estimate of soluble nitrogen using ANKOM fibre bags with a pore size of 20-25µm (Table 6.2). This indicates that 18.7% of water soluble nitrogen measured using the nylon bag is actually insoluble fine grass silage particles.

These fine insoluble silage particles (<1600µm) are also more likely to be in the liquid phase of rumen passage and assuming that they will be more slowly degraded than soluble protein components may contribute to the by-pass protein fraction. This is in agreement with other authors who have shown that up to 11% of the soluble protein fraction from ruminal bags can escape rumen degradation (Aufrère et al., 2001; Choi et al., 2002; Volden et al., 2002). The low efficiency of microbial capture of QDP assumed in the MP system therefore seems reasonable using data derived from nylon bag studies, but the assumption that QDP does not contribute to the by-pass protein fraction is questionable.

In contrast, the protein A fraction estimated using IVGPT was 23.7% lower than the SNAN concentration measured using ANKOM fibre bags (Table 6.3). Assuming that the ammonia content of fresh grass silage is immediately degraded, these results indicate that only 76.3% of the SNAN in grass silage is quickly degraded with 23.7% slowly degradable. The assumption made in the MP system that all soluble nitrogen is immediately and completely degraded to ammonia is therefore incorrect.

These results are supported by the findings of other authors who also observed a slowly degradable NAN (SDNAN) fraction (Chen et al., 1987). This SDNAN was attributed to the presence of free peptides which accumulate when the rate of peptide release is greater than the rate of peptide hydrolysis or microbial (Chen et al., 1987). These free peptides are
not readily utilised by rumen microbes and consequently there is a greater flow of peptides out of the rumen compared to soluble proteins and free amino acids, with a peak 1 hour after feeding and declining until 7 hours post feeding (Choi et al., 2002). Peptides were shown to account for 63.2% of all SNAN escaping rumen degradation, compared to soluble protein which accounted for 24.2% and free amino acids 12.6% (Choi et al., 2002). The rate limiting step in rumen proteolysis was therefore concluded to be the hydrolysis of peptides to amino acids rather than hydrolysis of soluble proteins to peptides or deamination of amino acids to ammonia (Choi et al., 2002). However, more recently it has been shown that the length of the peptide chain has a significant effect on the rate of rumen passage, with short chain peptides shown to be significantly more susceptible to rumen by-pass compared to long chain peptides, soluble proteins and free amino acids (Givens and Rulquin, 2003). A large proportion of the flow of amino acid-N to the duodenum maybe constituted of short chain peptides and could explain why only 76.3% of SNAN in this study was quickly degraded.

The results of this study indicate that the nylon bag technique and IVGPT techniques measure distinctly different protein fractions. Whereas the nylon bag technique measures protein disappearance, IVGPT measures the end products of fermentation resulting in significantly different degradation parameters estimates. The protein A fraction estimated using IVGPT was 40.2% lower (P<0.001) than that estimated using the nylon bag technique (Table 6.4) and was significantly correlated to the soluble nitrogen concentration (R²=77.9, P<0.001) (Table 6.5; Fig 6.3). In contrast, the A fraction estimated using the nylon bag technique showed no significant correlation with the soluble nitrogen concentration (R²=1.6, P>0.05) (Table 6.5; Fig 6.3), further indicating that the protein A fraction measured in the nylon bag technique does not measure soluble nitrogen but simply the proportion of dietary nitrogen that is small enough to pass out of the bag.

The advantage of using IVGPT to estimate the protein degradation profile of feeds is that it provides a measure of the rate of degradation of the quick protein A fraction and the lag before the slower protein B fraction is degraded, both of which are assumed to be instantaneous in the nylon bag technique. Although there was no significant difference in the estimated rate of degradation of the protein B fraction (P>0.05) or the proportion of undegraded protein, the estimated protein B fraction was 122% higher using the IVGPT technique that that estimated using the nylon bag technique (Table 6.4).
The large difference in the protein fraction estimates between the two analytical techniques had a significant effect on the estimated availability of feed protein in the rumen. The concentration of QDP was 52.8% lower (P<0.001) using IVGPT, which also predicted the concentration of SDP to be 104.5% higher (P<0.001) than that estimated using the nylon bag technique. Consequently, although the concentration of ERDP was estimated to be 16.7% lower (P<0.001) using IVGPT, the concentration of DUP was 155.7% higher (P<0.001) than that predicted using the nylon bag technique. This discrepancy is largely due to a fundamental flaw in the calculation of DUP when using the nylon bag technique, in which whilst assuming that microbes can only utilise 80% of QDP it simultaneously stated that no QDP is washed out of the rumen. This effectively means that 20% of QDP is unaccounted for, and based on the observations of other authors that QDP does contribute to the DUP fraction (Aufrère et al., 2001; Choi et al., 2002; Volden et al., 2002) means that the current approach may underestimate the concentration of DUP supplied by a diet and result in the overfeeding of supplementary dietary protein. It is therefore suggested that the current DUP equation within the MP system is modified to: DUP (%CP) = (0.9* (1 - ((QDP(%CP)*0.8) * CP(g/kgDM)) – (SDP (%CP)*CP(g/kgDM)) – 6.25)) / CP g/kgDM)

6.5.2 THE REPEATABILITY OF IVGPT IN ESTIMATING PROTEIN DEGRADATION PARAMETERS

The main limitation of the nylon bag technique is its poor repeatability, principally due to contamination of feed residue with microbial protein (Negi et al., 1988; Becker et al., 1995; Alexandrov, 1998) but also due to an imbalance of carbohydrate and protein availability in the ruminal fluid. The rate of degradation of feed protein in the rumen is not only dependent on the type and availability of nitrogen (Griswold et al., 1996; Russel, 1998) but also the type and availability of carbohydrate (Chamberlain et al., 1993; Dewhurst et al., 1999). Feeds with a high proportion of DUP are characteristically also low in rumen degradable carbohydrate (Christensen et al., 1993) and consequently if they are not incubated with a source of degradable carbohydrate, such as other feeds, the rate of protein degradation may be slow and therefore underestimated. This was illustrated during IVGPT by Cone and Gelder (1999) who demonstrated that the rate of casein fermentation was dependent on the rate of fermentation of other carbohydrate sources in the incubation mixture.

In contrast, using IVGPT microbial protein is removed from feed residue at the end of incubation using NDF solution and all feeds are incubated in the
presence of sucrose. Although IVPGT therefore measures the maximum rate of protein degradation it has the disadvantage that, unlike the nylon bag technique, there is no passage rate which in the rumen will have a significant effect on the availability of protein. Consequently protein degradation parameters estimated using IVGPT are best applied using a rumen model such as BioParaMilk© which simulates the dynamics of liquid and particulate passage through the rumen.

The results of this study indicated that IVPGT showed a reasonable degree of repeatability for all protein degradation parameters of grass silage, with the exception of the rate of degradation of the protein B fraction (T-value = 6.7) and the undegraded protein fraction (T-value = 3), which although lower than the optimum T-value of 10 had a standard deviation that was within the accuracy of the technique (±0.03) and therefore not significant (Table 6.6).
The nylon bag technique overestimates the concentration of soluble nitrogen by 23%.

The protein A fraction estimated using the nylon bag technique shows no correlation with the concentration of soluble nitrogen in grass silage and is significantly higher than that estimated using IVGPT, which is significantly correlated to the soluble NAN content.

The new IVGPT technique estimates grass silage to have a significantly lower concentration of QDP and ERDP and a significantly higher concentration of SDP and DUP compared to the estimates derived using the nylon bag technique.

Only 76.3% of soluble NAN in grass silage is quickly degraded with up to 37.5% being slowly degraded. The assumption of the MP system that soluble protein is immediately degraded is therefore incorrect.
Effect of sample preparation on the protein degradation parameters of grass silage, maize silage and wholecrop wheat using IVGPT
To best simulate in vivo conditions during in vitro evaluation, forages should be incubated in a form that is as similar as possible to that which is fed to the animal. The purpose of this study was to investigate the effect of sample processing on the estimated crude protein (CP) concentration of silages and to compare the protein degradation parameters of fresh and dried silages using IVGPT.

There was no significant loss of amino nitrogen from solutions of L-Alanine, L-Lysine and L-Proline when placed in a 60°C oven for 24 hours (P>0.05). It is therefore proposed that there is no loss of soluble free amino acids during oven drying of fresh silages.

There was a significant loss (P<0.001) of moisture from fresh samples when mechanically processed to simulate mastication. Failure to correct for this results in the overestimation of the concentration of crude protein in fresh material (P<0.001)

Using dried and milled material to determine the protein degradation parameters of silages, significantly underestimates the protein A fraction and the concentration of quickly degradable protein (QDP) in fresh grass silage (P<0.05; P<0.05) and maize silage (P<0.05; P<0.05). The use of dried and milled material also results in the overestimation of the protein B fraction and the concentration of effective rumen degradable protein (ERDP) in fresh grass silage (P<0.05; P<0.05) and maize silage (P<0.05). There was no significant effect of sample preparation on the protein degradation parameters of wholecrop wheat (P>0.05)

Multiple linear regression equations were developed to predict the protein degradation parameters of fresh forages from those measured in IVGPT for the same forages when dried and milled. Successful equations were developed to predict the proportion of quick A protein in fresh grass silage ($R^2=0.958$, $P<0.001$), the proportion of slow B protein in fresh grass silage ($R^2=0.958$, $P<0.001$), maize silage ($R^2=0.93$, $P<0.001$) and wholecrop ($R^2=0.985$, $P<0.001$) and the rate of slow B protein degradation ($C_s$) in fresh maize silage ($R^2=0.99$, $P<0.001$) and wholecrop ($R^2=0.98$, $P<0.01$).
The conclusion of this study is that dried and milled silages fail to provide a representative description of the protein degradation of fresh silages, under predicting QDP and over predicting SDP and ERDP.
7.2 INTRODUCTION

The protein value of feeds for ruminants is based on an estimate of the quantity of dietary or microbial protein absorbed from the small intestine. To determine the ability of the dietary protein to support maximal microbial growth and to give an indication of the level of dietary protein that will be available in the small intestine, the level and rate of degradation of ruminally available protein must be predicted.

Although many feeds are consumed in their fresh state, prior to analysis and incubation with rumen microbes most feeds are dried and milled through a 1 or 4 mm sieve. Processing feeds this way enables them to be easily stored and unlike fresh feeds which are very difficult to grind (Lopez et al., 1995), allows the incubation of a homogenous sample which increases the accuracy of measurement and reduces variation between samples.

Unfortunately, protein fractions are especially sensitive to the method of preservation (Abdalla et al., 1988). Using heat to dry silage and fermented feeds not only results in the loss of volatile amines and ammonia (Snyman and Joubert, 1992; Denium and Maassen, 1994) but also decreases protein solubility (Abdalla et al., 1988) and increases the fraction of insoluble protein (Goering and Waldo, 1974). Heating also causes protein condensation which at medium drying temperatures can result in the denaturing of insoluble proteins and decrease the rate of degradation, but at high temperatures (>100°C) can also cause carbohydrate and protein fractions to combine and form Maillard products which are highly indigestible (Van Soest and Mason, 1991; Deinum and Maassen, 1994). Chamley et al., (1990) showed that drying forages at 200°C significantly reduced the degradability of forage protein and increased the observed level of by-pass protein.

Immature forage legumes and grasses contain significantly more degradable protein which includes a high level of non-protein nitrogen (NPN), primarily composed of ammonia, nitrate, amines, amides and free amino acids. As the plant matures and plant protein synthesis advances, the cell wall matrix becomes more complex and renders forage protein less accessible to rumen microbes and thus less digestible (Hoffman et al., 1993). During ensiling there is a significant change in the forage nitrogen fractions as microbial and plant proteases (Zhu et al., 1999) break down forage protein into smaller soluble and more degradable fractions, principally free amino acids and
ammonia (Givens and Rulquin, 2003), resulting in only 9-15% of macro
forage protein (>10kDa) remaining after fermentation (Messman et al., 1994).

For those immature forages and silages where a relatively large proportion of
the degradable protein may be lost or altered during the drying process,
analysis of the rate and extent of protein digestion of feeds after they have
been dried and milled may therefore be misleading (Lowman et al., 2002).

The aims of this study were to (1) assess the effect of sample preparation on
the crude protein determination of silages and (2) to compare the protein
degradation parameters of fresh and dried silages estimated using IVGPT.
7.3 MATERIAL AND METHODS

7.3.1 EXPERIMENTAL TREATMENTS

EXPERIMENT 1

This experiment was designed to study the effect of oven drying and simulated mastication on the estimated crude protein concentration of silages.

EXPERIMENT 2

This experiment was designed to compare the protein fermentation profile of fresh and dried silages estimated using a modified in vitro gas production technique (IVGPT) and to develop equations to predict the protein degradation parameters of fresh silages using dried material.

7.3.2 PROXIMATE ANALYSIS

Samples of 10 grass silages made from predominantly perennial ryegrass swards, 9 maize silages, and 9 wholecrop wheat samples, all cut in 2003, were obtained from commercial farms throughout the United Kingdom (UK) by Bioparametrics Ltd.

The DM content of each forage sample was measured by drying approximately 100g of fresh material in a 60°C oven for 24 hours. The dried samples were then milled through a 1mm sieve using a Retsch© mill, analysed for NDF (Van Soest et al., 1991) and ADF using ANKOM F57© fibre bags and crude protein (CP) using the standard Kjedahl technique. See Appendix A.1 for details of the methodology used. The lactic acid (g/kgDM), total volatile fatty acid (VFA) (g/kgDM), ammonia (g/kgDM) content and pH of each of the silages was estimated using NIRS (Foss NIRSystem 6500).

To simulate mastication, the fresh silage samples were frozen overnight and then approximately 15g of the frozen fresh material was chopped in a Moulinex Ovatio 3 Duo blender at 1450rpm for 15 seconds.

The CP concentration of the fresh silage was measured by digesting the equivalent of 500mgDM of fresh material using the standard Kjedahl technique.
Corrected Fresh CP (g/kgDM) = Fresh CP (g/kgDM) - (Ammonia N (g/kgDM) * 6.25)

Unaccounted CP (g/kgDM) = Fresh CP (g/kgDM) – (Ammonia N (g/kgDM) * 6.25) – Dry CP (g/kgDM)

7.3.3 AMINO ACID-N LOSS DURING OVEN DRYING

Standard amino acid solutions were prepared: L-Alanine (2000mg/l), L-Lysine (1000mg/l) and L-Proline (2000mg/l).

For each amino acid solution, an aluminium dish was weighed (W₁) and 250ml of the amino acid solution added. The aluminium dish and the solution were weighed (W₂) and placed in a 60°C oven. After 24 hours, the aluminium dishes were removed from the oven, allowed to cool in a dessicator and re-weighed (W₃).

The nitrogen concentration of each solution was measured before (N₁) and after (N₂) oven drying using a FOSS FIAstar 5000 Analyzer fitted with a 5027 sampler unit. The final nitrogen concentration was calculated by applying the formula:

\[
\text{Final (mgN/L)} = \frac{(N_2 \times ((250 - (W_2 - W_3)) / 250)) \times (1000 \text{ / tested volume (ml)})}{1}
\]

7.3.4 PREPARATION OF ZEOLITE

The zeolite (Clinoptilolite) was purchased from Euremica Environmental Ltd and had a particle size of 2-3 mm. Because the zeolite was received in its raw state it was first processed and washed to remove any small particles and organic material that could contaminate the filtrate (see Appendix A.1.5)

7.3.5 ESTIMATING THE PROTEIN DEGRADATION PROFILE OF DRIED AND FRESH SILAGES USING IVGPT

After simulated mastication, the fresh samples were allowed to defrost at room temperature for twenty minutes. Glass syringes (100ml) were primed with approximately 100mg of sucrose and then a weight of silage equating to 78.4 mgN/L was added in triplicate into the syringes, which were then placed in the fridge until the morning of incubation:

\[
\text{Dry silage (mg)} = \frac{((1000 / CP \text{ (g/kgDM)}) \times 0.1875 \text{ (mgCP/30ml)}) \times 78.4 \text{ mgN/L}}{1}
\]
Fresh silage (mg) = \(((1000 / CP \text{ (g/kgDM)}) \times 0.1875 \text{ (mgCP/30ml)}) \times 78.4 \text{ mgN/L}) \\ \times (1000/DM \text{ (g/kgDM)})

Three additional syringes were also created and placed in the fridge but contained no samples.

On the morning of inoculation and prior to collecting the rumen liquor, the syringes containing the samples, along with ‘blanks’ containing no substrate, were placed in a 39°C water bath.

Rumen liquor was collected from sheep kept outside during summer and grazing predominantly perennial ryegrass with no access to supplemental feeds. On the morning of inoculation, individual sheep were collected from the pasture at approximately 09:30, slaughtered and the rumen and reticulum removed and placed in a thermos box to keep warm whilst travelling back to the laboratory. At the laboratory the rumen contents were strained through two layers of 250 µm netting and three layers of muslin, and then stored in a thermos flask gassed with CO₂.

The microbial activity of the rumen liquor before filtration was assessed by measuring its absorbance at 600 nm (Nagadi et al., 2000). The dilution ratio with anaerobic medium was then calculated to ensure that the inoculum had an absorbance at 600 nm of 0.111 - 0.134.

Approximately 1 litre of rumen liquor was filtered through 400g of zeolite at a flow rate of 8.92 BV/h (see Appendix A.1.5), stored in a thermos flask and gassed with CO₂.

The appropriate volume of nitrogen free ABM (Menke and Steingass, 1988) was prepared (see Appendix A.1.5), placed in a 39°C water bath and saturated with CO₂. When the medium reached 39°C, the filtered rumen liquor was added and the inoculum was gassed with CO₂ for a further 5 minutes. Using an automatic dispensing pump, 30 ml of inoculum was added to each fermentation syringe before it was returned to the water bath. Measurement of in vitro gas production were performed as described by Jessop and Herrero (1996), with cumulative gas production in each syringe read every half hour for the first 3 hours, then hourly until 6 hours and thereafter at 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 56, 64, and 72h. Syringes were manually shaken at each reading.
After 72h of incubation the residual nitrogen concentration was measured by first emptying each syringe into 200ml flat medicinal bottles containing 15ml of NDF solution and autoclaving at 105°C for 1 hour to solublise microbial protein. The contents of each bottle were filtered through Whatman 54 Hardened filter paper and the residual nitrogen measured using a standard Kjeldhal technique (see Appendix A.1.5).

7.3.6 INTERPRETATION OF GAS PRODUCTION DATA

The protein degradation profile of the silage was determined by applying the analytical procedure described in Appendix A1.5. This initially describes the protein degradation of grass silage in terms of the proportion (A) and rate (Cₐ) of quickly fermentable protein and then after a lag, the proportion (B) and rate (Cₐ) of slowly fermentable protein (SDP). Assuming an outflow rate (Kp) of 0.08/h (AFRC, 1993) these degradation parameters are then used to estimate the effective protein degradability of each grass silage in terms of the proportion of quickly (QDP) and slowly (SDP) degradable protein, the effective rumen degradable protein (ERDP) and digestible undegraded protein (DUP).

7.3.7 STATISTICAL ANALYSIS

Using the Minitab statistical package (2000), paired T-tests were used to compare the protein degradation parameters of the dried and fresh silages during IVGPT, the effect of oven drying on the level of amino acid nitrogen and the effect of simulated mastication on silage DM. Analysis of variance (ANOVA) using a general linear model (GLM) was used to compare the effect of silage type on the estimated protein degradation parameters.

Linear regression was used to assess the relationship between estimated DM and CP determination of silage.

Multiple linear regressions were used to predict the protein degradation parameters of fresh silages using proximate results and the protein gas production parameters of dried silages as independent variables. The independent variables used for each forage type was selected using the ‘best subset’ function within the statistical software (Minitab, 2000). Outliers were removed using the ‘unusual observation function’ within the Minitab (2000), which removes observations with a standardized residual greater than 2.
7.4 RESULTS

7.4.1 EXPERIMENT 1 – EFFECT OF SAMPLE PREPARATION ON CRUDE PROTEIN DETERMINATION

7.4.1.1 PROXIMATE ANALYSIS

The proximate analyses of dried and fresh silages studied are presented in Table 7.1 and Table 7.2.

Table 7.1 Proximate Analyses of grass silage (n=10), maize silage (n=9) and wholecrop wheat (n=9)

<table>
<thead>
<tr>
<th></th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Grass</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM (g/kg)</td>
<td>299.9</td>
<td>93.2</td>
</tr>
<tr>
<td>ADF (g/kgDM)</td>
<td>304.5</td>
<td>46.9</td>
</tr>
<tr>
<td>NDF (g/kgDM)</td>
<td>469.9</td>
<td>63.7</td>
</tr>
<tr>
<td><strong>Silage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry CP (g/kgDM)</td>
<td>117.8</td>
<td>26.0</td>
</tr>
<tr>
<td>Fresh CP (g/kgDM)</td>
<td>147.8</td>
<td>29.1</td>
</tr>
<tr>
<td>Ammonia-N (g/kgDM)</td>
<td>0.92</td>
<td>0.38</td>
</tr>
<tr>
<td>Unaccounted CP (g/kgDM)</td>
<td>24.3</td>
<td>10.8</td>
</tr>
<tr>
<td><strong>Maize</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM (g/kg)</td>
<td>278.2</td>
<td>30.4</td>
</tr>
<tr>
<td>ADF (g/kgDM)</td>
<td>264.5</td>
<td>23.0</td>
</tr>
<tr>
<td>NDF (g/kgDM)</td>
<td>388.1</td>
<td>29.1</td>
</tr>
<tr>
<td><strong>Silage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry CP (g/kgDM)</td>
<td>71.9</td>
<td>10.4</td>
</tr>
<tr>
<td>Fresh CP (g/kgDM)</td>
<td>85.3</td>
<td>13.4</td>
</tr>
<tr>
<td>Ammonia-N (g/kgDM)</td>
<td>0.83</td>
<td>0.12</td>
</tr>
<tr>
<td>Unaccounted CP (g/kgDM)</td>
<td>8.6</td>
<td>6.7</td>
</tr>
<tr>
<td><strong>Wheat</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM (g/kg)</td>
<td>520.5</td>
<td>146.5</td>
</tr>
<tr>
<td>ADF (g/kgDM)</td>
<td>340.3</td>
<td>75.6</td>
</tr>
<tr>
<td>NDF (g/kgDM)</td>
<td>454.2</td>
<td>81.9</td>
</tr>
<tr>
<td>CP (g/kgDM)</td>
<td>80.1</td>
<td>26.1</td>
</tr>
<tr>
<td>Fresh CP (g/kgDM)</td>
<td>109.5</td>
<td>28.8</td>
</tr>
<tr>
<td>Ammonia-N (g/kgDM)</td>
<td>0.69</td>
<td>0.49</td>
</tr>
<tr>
<td>Unaccounted CP (g/kgDM)</td>
<td>25.0</td>
<td>13.1</td>
</tr>
</tbody>
</table>

The concentration of crude protein (CP) in the dried silages was significantly higher in grass silage compared to maize silage and wholecrop wheat (P<0.05), which were not significantly different (P>0.05). In fresh silage, the concentration of CP was significantly higher in grass silage compared to wholecrop wheat (P<0.05), which was significantly higher than maize silage
(P<0.05) (Fig 7.2). After accounting for the ammonia lost during oven drying, the concentration of unaccountable CP was significantly lower (P<0.05) in maize silage compared to grass silage and wholecrop wheat, which were not significantly different (P>0.05)

Table 7.2 Comparison between the proximate analyses values of grass silage, maize silage and wholecrop wheat. Standard deviation values are shown in brackets. For each silage type, means with different superscripts within the same row are significantly different (P<0.05).

<table>
<thead>
<tr>
<th>Type</th>
<th>Grass Silage</th>
<th>Maize Silage</th>
<th>Wholecrop Wheat</th>
<th>Type Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>10</td>
<td>9</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>DM (g/kg)</td>
<td>288.0 (90.5)a</td>
<td>278.2 (30.4)a</td>
<td>520.5 (146.5)b</td>
<td>***</td>
</tr>
<tr>
<td>ADF (g/kgDM)</td>
<td>308.5 (47.9)a</td>
<td>264.5 (23.0)b</td>
<td>340.4 (75.6)a</td>
<td>*</td>
</tr>
<tr>
<td>NDF (g/kgDM)</td>
<td>474.9 (65.2)a</td>
<td>388.1 (29.1)b</td>
<td>454.2 (81.9)a</td>
<td>*</td>
</tr>
<tr>
<td>Dry CP (g/kgDM)</td>
<td>122.4 (22.8)a</td>
<td>71.9 (10.4)b</td>
<td>80.1 (26.1)b</td>
<td>***</td>
</tr>
<tr>
<td>Fresh CP (g/kgDM)</td>
<td>151.9 (27.6)a</td>
<td>85.3 (13.4)b</td>
<td>109.5 (28.8)c</td>
<td>***</td>
</tr>
<tr>
<td>Ammonia-N (g/kgDM)</td>
<td>0.96 (0.39)</td>
<td>0.83 (0.12)</td>
<td>0.26 (0.72)</td>
<td>NS</td>
</tr>
<tr>
<td>Unaccounted CP (g/kgDM)</td>
<td>23.7 (11.2)a</td>
<td>8.6 (6.7)b</td>
<td>25.0 (13.1)a</td>
<td>**</td>
</tr>
</tbody>
</table>

NS P>0.05  *P<0.05  **P<0.01  ***P<0.001

The concentration of unaccountable CP in the fresh silages is present in Table 7.3 and illustrated in Fig 7.1.

The CP concentration was estimated to be 25.5% higher in fresh grass silage, 18.6% higher in fresh maize silage and 36.7% higher in fresh wholecrop wheat, compared to the dried and milled material. After accounting for the ammonia assumed to be lost during oven drying, the CP concentration of the fresh material was still 20.5% higher in grass silage, 11.4% higher in maize silage and 31.2% higher in wholecrop wheat (Table 7.3)
Table 7.3 Comparison of the crude protein concentration (g/kgDM) of grass silage (n=10), maize silage (n=9) and wholecrop wheat (n=9) when analysed dried and milled, fresh and chopped or fresh and chopped and corrected for ammonia-N. Standard deviation values are shown in brackets.

<table>
<thead>
<tr>
<th>Type</th>
<th>Dry</th>
<th>Fresh</th>
<th>Fresh Corrected</th>
<th>Sig.</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass silage</td>
<td>117.8 (26.0)</td>
<td>147.8 (29.1)</td>
<td>142.0 (27.7)</td>
<td>***</td>
<td>+25.5</td>
</tr>
<tr>
<td>Maize silage</td>
<td>71.9 (10.4)</td>
<td>85.3 (13.4)</td>
<td>80.1 (12.9)</td>
<td>***</td>
<td>+18.6</td>
</tr>
<tr>
<td>Wholecrop wheat</td>
<td>80.1 (26.1)</td>
<td>109.5 (28.8)</td>
<td>105.1 (28.1)</td>
<td>***</td>
<td>+36.7</td>
</tr>
</tbody>
</table>

NS P>0.05 *P<0.05 **P<0.01 ***P<0.001

7.4.1.2 AMINO ACID-N LOSS DURING OVEN DRYING

There was no significant loss of amino acid nitrogen from solutions of L-Alanine (P>0.05), L-Lysine (P>0.05) or L-Proline (P>0.05) when incubated in a 60°C oven for 24 hours.

7.4.1.3 EFFECT OF DM ON THE CP ESTIMATION OF FRESH GRASS SILAGE

The effect of simulated mastication on the DM content of grass silage is presented in Table 7.4. The linear relationship between DM and the estimated concentration of CP is presented in Table 7.10.

The DM content of grass silage after simulated mastication was 13.9% higher (P<0.001) than before (Table 7.4). There was a negative linear relationship between the increase in silage DM after simulated mastication and the estimated CP concentration (R² = 99.8, P<0.001) (Table 7.5).
Table 7.4 Comparison of the DM concentration (g/kg) of grass silage (n=10) determined on the day of arrival and after simulated mastication treatment. Standard deviation values are shown in brackets.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pre-freezing</th>
<th>Post-treatment</th>
<th>Sig.</th>
<th>Difference (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DM (g/kg)</td>
<td>299.9 (93.2)</td>
<td>341.8 (89.4)</td>
<td>***</td>
<td>+13.9</td>
</tr>
</tbody>
</table>

NS P>0.05 *P<0.05 **P<0.01 ***P<0.001

Table 7.5 Linear relationship between the DM (g/kg) change (%) of fresh grass silage (n=10) after simulated mastication treatment and the change (%) in the estimated CP concentration.

<table>
<thead>
<tr>
<th>Method Type</th>
<th>n</th>
<th>Equation</th>
<th>R²</th>
<th>SEE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP (% Change)</td>
<td>10</td>
<td>-0.7025 * DM (% Difference) + 1.6597</td>
<td>0.998</td>
<td>1.14</td>
<td>***</td>
</tr>
</tbody>
</table>

NS P>0.05 *P<0.05 **P<0.01 ***P<0.001

After correcting for the ammonia lost during oven drying and the increase in DM following simulated mastication there was no significant difference (P>0.05) in the estimated CP concentration between dry and fresh grass silage (Table 7.6)

Table 7.6 Comparison of the crude protein concentration (g/kgDM) of grass silage (n=10), maize silage (n=9) and wholecrop wheat (n=9) when analysed dried and fresh corrected for either ammonia-N loss or ammonia-N loss and DM change. Standard deviation values are shown in brackets.

<table>
<thead>
<tr>
<th>Type</th>
<th>Dry</th>
<th>Fresh NH₃ Corrected</th>
<th>Fresh NH₃ &amp; DM Corrected</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass silage</td>
<td>117.8 (26.0)</td>
<td>142.0 (27.7)</td>
<td>126.7 (25.0)</td>
<td>***</td>
</tr>
</tbody>
</table>

NS P>0.05 *P<0.05 **P<0.01 ***P<0.001
7.4.2 EXPERIMENT 2 – COMPARISON OF THE ESTIMATED PROTEIN DEGRADATION PARAMETERS OF FRESH AND DRIED SILAGE

7.4.2.1 PROXIMATE ANALYSIS

The proximate analyses results for the silage samples studied are presented in Table 7.7.

Table 7.7 Proximate Analyses of dry and milled and fresh and chopped grass silage, maize silage and wholecrop wheat. Standard deviation values are shown in brackets.

<table>
<thead>
<tr>
<th>Type</th>
<th>Grass silage</th>
<th>Maize silage</th>
<th>Wholecrop Wheat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>DM (g/kg)</td>
<td>322.0 (100.1)</td>
<td>283.1 (35.0)</td>
<td>517.7 (122.5)</td>
</tr>
<tr>
<td>NDF (g/kgDM)</td>
<td>444.1 (34.7)</td>
<td>381.4 (26.2)</td>
<td>457.5 (100.4)</td>
</tr>
<tr>
<td>Dry CP (g/kgDM)</td>
<td>118.6 (27.0)</td>
<td>71.67 (12.95)</td>
<td>78.39 (22.58)</td>
</tr>
<tr>
<td>Fresh CP (g/kgDM)</td>
<td>143.8 (29.8)</td>
<td>88.24 (15.31)</td>
<td>100.3 (28.70)</td>
</tr>
</tbody>
</table>

7.4.2.2 ESTIMATED PROTEIN DEGRADATION PARAMETERS OF FRESH AND DRIED FORAGES USING IVDMD

The estimated protein degradation parameters of fresh and dried grass silage, maize silage and wholecrop wheat are presented in Table 7.8 and Table 7.9 and illustrated in Fig 7.1.

Compared to dried grass silage, the protein A fraction was 5.6% higher in fresh grass silage (P<0.05), which also had a 6.2% lower protein B fraction (P<0.05). Consequently, it was estimated that fresh grass silage provided 7.2% (P<0.05) more QDP and 3% more ERDP (P<0.01) than dried grass silage. There was no significant difference (P>0.05) between dried and fresh grass silage for any other protein degradation parameter (Table 7.8).

Compared to dried maize silage, the protein A fraction was 8.7% higher in fresh maize silage (P<0.05). The protein B fraction was also 5.8% lower (P<0.05) in fresh maize silage and shown to be more quickly degraded (P<0.01). Fresh maize silage supplied 10.0% more (P<0.05) QDP and 4.3% more ERDP (P<0.05) compared to dried maize silage. There was no significant difference (P>0.05) between dried and fresh maize silage for any other protein degradation parameter.
There was no significant difference (P>0.05) in the protein degradation parameters between dried and fresh wholecrop wheat but fresh wholecrop wheat was estimated to supply 10.6% less SDP than dried material (P<0.05).

In both its dry and fresh state grass silage had a significantly higher protein A fraction (P<0.05) and a significantly lower protein B fraction (P<0.05) compared to maize silage and wholecrop wheat, which were not significantly different (P>0.05) (Table 7.9). The rate of degradation of the protein B fraction was also significantly higher (P<0.05) in both dried and fresh grass silage compared to maize silage and wholecrop wheat, which were not significantly different (P>0.05). There was no significant difference in the lag (P>0.05) or proportion of undegraded protein between grass silage, maize silage or wholecrop wheat, either fresh or dry (P>0.05).

In both fresh and dried material there was no significant difference (P>0.05) in the proportion of SDP or DUP supplied by grass silage, maize silage or wholecrop wheat, but grass silage contained a significantly higher (P<0.05) proportion of QDP and ERDP compared to maize silage and wholecrop wheat, which were not significantly different (P>0.05).

![Graph](image)

**Fig 7.1** Estimated protein degradation profile of dried grass silage (white square), fresh grass silage (black square), dry maize silage (black circle), fresh maize silage (white circle), dry wholecrop wheat (white triangle) and fresh wholecrop wheat (black triangle). Samples were incubated in triplicate (n=3).
Table 7.8 Comparison of the estimated protein degradation parameters of dried and fresh grass silage \((n=5)\), maize silage \((n=5)\) and wholecrop wheat \((n=5)\) determined using IVGPT. All samples were incubated in triplicate \((n=3)\). Standard deviation values are shown in brackets.

| Type    | Parameters | Dry & Milled | Fresh & Chop’d | Sig. | Difference (%)
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass</td>
<td>A (%CP)</td>
<td>0.463 (0.085)</td>
<td>0.489 (0.047)</td>
<td>*</td>
<td>+5.6</td>
</tr>
<tr>
<td></td>
<td>C_d (h⁻¹)</td>
<td>0.432 (0.052)</td>
<td>0.469 (0.062)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B (%CP)</td>
<td>0.418 (0.085)</td>
<td>0.392 (0.047)</td>
<td>*</td>
<td>-6.2</td>
</tr>
<tr>
<td></td>
<td>C_b (h⁻¹)</td>
<td>0.0622 (0.0053)</td>
<td>0.0629 (0.0042)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Silage</td>
<td>Lag (h)</td>
<td>0.733 (0.082)</td>
<td>0.717 (0.077)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>U (%CP)</td>
<td>0.119 (0.00)</td>
<td>0.119 (0.00)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>QDP @ 0.08/h (%CP)</td>
<td>0.389 (0.066)</td>
<td>0.417 (0.042)</td>
<td>*</td>
<td>+7.2</td>
</tr>
<tr>
<td></td>
<td>SDP @ 0.08/h (%CP)</td>
<td>0.173 (0.041)</td>
<td>0.163 (0.019)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERDP @ 0.08/h (%CP)</td>
<td>0.562 (0.026)</td>
<td>0.579 (0.027)</td>
<td>**</td>
<td>+3.0</td>
</tr>
<tr>
<td></td>
<td>DUP @ 0.08/h (%CP)</td>
<td>0.339 (0.019)</td>
<td>0.333 (0.018)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Maize</td>
<td>A (%CP)</td>
<td>0.343 (0.019)</td>
<td>0.373 (0.025)</td>
<td>*</td>
<td>+8.7</td>
</tr>
<tr>
<td></td>
<td>C_d (h⁻¹)</td>
<td>0.5889 (0.087)</td>
<td>0.6307 (0.0626)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B (%CP)</td>
<td>0.514 (0.054)</td>
<td>0.484 (0.032)</td>
<td>*</td>
<td>-5.8</td>
</tr>
<tr>
<td></td>
<td>C_b (h⁻¹)</td>
<td>0.0492 (0.0085)</td>
<td>0.0509 (0.0093)</td>
<td>**</td>
<td>+3.5</td>
</tr>
<tr>
<td>Silage</td>
<td>Lag (h)</td>
<td>0.693 (0.014)</td>
<td>0.716 (0.055)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>U (%CP)</td>
<td>0.143 (0.053)</td>
<td>0.143 (0.053)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>QDP @ 0.08/h (%CP)</td>
<td>0.301 (0.018)</td>
<td>0.331 (0.026)</td>
<td>*</td>
<td>+10.0</td>
</tr>
<tr>
<td></td>
<td>SDP @ 0.08/h (%CP)</td>
<td>0.185 (0.036)</td>
<td>0.177 (0.028)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERDP @ 0.08/h (%CP)</td>
<td>0.487 (0.042)</td>
<td>0.508 (0.052)</td>
<td>*</td>
<td>+4.3</td>
</tr>
<tr>
<td></td>
<td>DUP @ 0.08/h (%CP)</td>
<td>0.373 (0.045)</td>
<td>0.370 (0.049)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>A (%CP)</td>
<td>0.322 (0.038)</td>
<td>0.323 (0.078)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C_d (h⁻¹)</td>
<td>0.4859 (0.0919)</td>
<td>0.4844 (0.1351)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B (%CP)</td>
<td>0.499 (0.102)</td>
<td>0.468 (0.148)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C_b (h⁻¹)</td>
<td>0.0587 (0.0058)</td>
<td>0.0577 (0.0064)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lag (h)</td>
<td>0.571 (0.259)</td>
<td>0.98 (0.43)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>U (%CP)</td>
<td>0.167 (0.065)</td>
<td>0.191 (0.106)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>QDP @ 0.08/h (%CP)</td>
<td>0.275 (0.031)</td>
<td>0.277 (0.077)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SDP @ 0.08/h (%CP)</td>
<td>0.199 (0.033)</td>
<td>0.178 (0.045)</td>
<td>*</td>
<td>-10.6%</td>
</tr>
<tr>
<td></td>
<td>ERDP @ 0.08/h (%CP)</td>
<td>0.475 (0.021)</td>
<td>0.455 (0.068)</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DUP @ 0.08/h (%CP)</td>
<td>0.384 (0.027)</td>
<td>0.421 (0.066)</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

NS: \(P>0.05\) *: \(P<0.05\) **: \(P<0.01\) ***: \(P<0.001\)

140
Table 7.9 Comparison of the estimated protein degradation parameters of dried and fresh grass silage (n=5), maize silage (n=5) and wholecrop wheat (n=5) determined using IVGPT. All samples were incubated in triplicate (n=3). Standard deviation values are shown in brackets. Silage types with different superscripts in the same row are significantly different (P>0.05).

<table>
<thead>
<tr>
<th>Type</th>
<th>Parameters</th>
<th>Grass silage</th>
<th>Maize silage</th>
<th>Wholecrop Wheat</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry &amp; Milled</td>
<td>A (%CP)</td>
<td>0.463 (0.085)a</td>
<td>0.343 (0.191)b</td>
<td>0.322 (0.038)b</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Ca (h⁻¹)</td>
<td>0.4321 (0.0524)</td>
<td>0.5889 (0.0872)</td>
<td>0.4859 (0.0919)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B (%CP)</td>
<td>0.418 (0.085)a</td>
<td>0.514 (0.054)b</td>
<td>0.499 (0.102)b</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Cb (h⁻¹)</td>
<td>0.0622 (0.0053)a</td>
<td>0.0492 (0.0085)b</td>
<td>0.0587 (0.0058)b</td>
<td>*</td>
</tr>
<tr>
<td>Lag (h)</td>
<td>QDP (%CP)</td>
<td>0.389 (0.066)a</td>
<td>0.301 (0.018)b</td>
<td>0.275 (0.031)b</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>SDP (%CP)</td>
<td>0.173 (0.041)</td>
<td>0.185 (0.036)</td>
<td>0.199 (0.033)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>ERDP (%CP)</td>
<td>0.562 (0.026)a</td>
<td>0.487 (0.042)b</td>
<td>0.475 (0.021)b</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>DUP (%CP)</td>
<td>0.339 (0.019)</td>
<td>0.373 (0.045)</td>
<td>0.384 (0.027)</td>
<td>NS</td>
</tr>
<tr>
<td>Fresh &amp; Chopped</td>
<td>A (%CP)</td>
<td>0.489 (0.047)a</td>
<td>0.373 (0.025)b</td>
<td>0.341 (0.079)b</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Ca (h⁻¹)</td>
<td>0.4690 (0.0624)</td>
<td>0.6307 (0.0626)</td>
<td>0.5087 (0.1291)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B (%CP)</td>
<td>0.392 (0.0471)a</td>
<td>0.4839 (0.0145)b</td>
<td>0.4683 (0.1282)b</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>Cb (h⁻¹)</td>
<td>0.0629 (0.0042)a</td>
<td>0.0509 (0.0093)b</td>
<td>0.0583 (0.0056)b</td>
<td>*</td>
</tr>
<tr>
<td>Lag (h)</td>
<td>QDP (%CP)</td>
<td>0.417 (0.042)a</td>
<td>0.331 (0.026)b</td>
<td>0.294 (0.077)b</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>SDP (%CP)</td>
<td>0.163 (0.019)</td>
<td>0.177 (0.028)</td>
<td>0.180 (0.039)</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>ERDP (%CP)</td>
<td>0.579 (0.027)a</td>
<td>0.508 (0.052)b</td>
<td>0.475 (0.074)b</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>DUP (%CP)</td>
<td>0.333 (0.018)</td>
<td>0.370 (0.048)</td>
<td>0.409 (0.063)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS P>0.05 *P<0.05 **P<0.01 ***P<0.001

7.4.2.3 PREDICTING THE PROTEIN DEGRADATION PARAMETERS OF FRESH SILAGES USING DRIED MATERIAL

Assuming that the rate of fermentation of quick A protein is 0.3 h⁻¹, predicting the protein degradation parameters of fresh silages when using dried material in IVGPT is only partially successful using multiple regression equations. The proportion of quick A protein in fresh silage can be predicted for grass silage (R²=0.958, P<0.001) but not for maize silage or wholecrop wheat (P>0.05). The proportion of slow B protein can be predicted for grass
silage (R²=0.958, P<0.001), maize silage (R²=0.93, P<0.001) and wholecrop (R²=0.985, P<0.001), but the rate of degradation of B (Cb) can only be predicted for maize silage (R²=0.99, P<0.001) and wholecrop (R²=0.98, P<0.01) but not grass silage (P>0.05). The protein lag cannot be predicted for any of the comparable forages (P>0.05) (Table 7.10).

Table 7.10 Multiple linear regression equations to predict the protein degradation parameters of fresh grass silage, maize silage and wholecrop wheat from standard analysis values and the estimated gas production parameters of 220mgDM of dried grass silage (n=5), maize silage (n=5) and wholecrop wheat (n=5) when incubated in ovine rumen liquor. SEE is the standard error (+/-) of each estimated parameter.

<table>
<thead>
<tr>
<th>Type</th>
<th>Equation</th>
<th>R²</th>
<th>SEE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass</td>
<td>A (prop CP) 0.237 + 0.543 a (prop CP)</td>
<td>0.958</td>
<td>0.011</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Ca (l/h) 0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>B (prop CP) 0.165 + 0.543 b (prop CP)</td>
<td>0.958</td>
<td>0.011</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Cb (l/h) 0.0416 + 0.512 cb (l/h) - 0.000033 DM (g/kg)</td>
<td>0.577</td>
<td>0.0039</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Lag (h) 2.38 - 0.632 lag (h) - 0.0027 NDF (g/kgDM)</td>
<td>0.805</td>
<td>0.048</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>A (prop CP) -1.24 + 2.43 a (prop CP) + 0.00109 DM (g/kg) + 0.00122 NDF (g/kgDM)</td>
<td>0.628</td>
<td>0.03</td>
<td>NS</td>
</tr>
<tr>
<td>Maize</td>
<td>Ca (l/h) 0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silage</td>
<td>B (prop CP) 0.187 + 0.578 b (prop CP)</td>
<td>0.939</td>
<td>0.009</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Cb (l/h) -0.00326 + 1.1 cb (l/h) - 0.000033 DM (g/kgDM)</td>
<td>0.999</td>
<td>0.0036</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Lag (h) 0.95 + 3.36 lag (h) - 0.00175 NDF (g/kgDM)</td>
<td>0.708</td>
<td>0.04</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>A (prop CP) 0.409 + 0.628 a (prop CP) - 0.00059 DM (g/kg)</td>
<td>0.99</td>
<td>0.014</td>
<td>NS</td>
</tr>
<tr>
<td>Wholecrop</td>
<td>Ca (l/h) 0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wheat</td>
<td>B (prop CP) 0.252 + 1.44 b (prop CP)</td>
<td>0.985</td>
<td>0.022</td>
<td>***</td>
</tr>
<tr>
<td></td>
<td>Cb (l/h) -0.00613 + 1.09 cb (l/h)</td>
<td>0.98</td>
<td>0.0011</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>Lag (h) -1.81 + 1.34 lag (h) + 0.0045 DM (g/DM)</td>
<td>0.993</td>
<td>0.062</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS P>0.05 *P<0.05 **P<0.01 ***P<0.001
7.5 DISCUSSION

7.5.1 EFFECT OF SAMPLE PREPARATION ON CRUDE PROTEIN DETERMINATION

In an attempt to create a homogenous sample the fresh samples in this study were chopped in an electric blender prior to analysis. After accounting for ammonia which was assumed to be lost during the drying procedure, there was still a significant difference in the estimated crude protein (CP) concentration between fresh and dried silages (Table 7.3). The CP concentration was 20.5% higher in fresh grass silage (P<0.001), 11.4% higher in fresh maize silage (P<0.01) and 31.2% higher in fresh wholecrop wheat (P<0.001) compared to dried material (Table 7.3). This unaccountable presence of CP indicates that there maybe an additional source of protein loss during the drying process and/or an error in the analytical procedure.

One possibility is the loss of soluble nitrogen during oven drying, specifically aromatic and heterocyclic free amino acids such as L-proline. Free amino acids can contribute 25-42% of the total nitrogen concentration of silage depending on the forage type (Givens and Rulquin, 2003) and therefore account for a substantial proportion of silage CP. Unfortunately analysis of the amino acid loss from silage prior to and after oven drying was not possible due to a lack of equipment, but this study found no significant (P>0.05) loss of amino acid nitrogen during the oven incubation of solutions of L-Proline, L-Alanine or L-Lysine. It was therefore concluded that soluble free amino acids are not lost during oven drying.

Determining the CP concentration of fresh forages is reliant on an accurate estimate of the DM content on the analysed sample. Unfortunately this study indicated that processing fresh grass silage in an electric blender to simulate mastication resulted in a significant increase in the DM of the final sample (P<0.001) (Table 7.4). Even though the fresh samples were blended in a frozen state to reduce the loss of soluble components, a soluble residue was still observed in the blending bowl after processing. In addition, although every attempt was made to process the samples quickly, the samples may have continued to lose moisture at room temperature whilst other samples were being processed. Failure to correct for this difference in DM results in the overestimation of the CP concentration of the processed fresh sample (Table 7.5). After correcting for the ammonia lost during oven drying and the increase in DM after simulated mastication there was no significant difference in the estimated CP concentration between fresh and dry grass
silage (P>0.05) (Table 7.6). The loss of moisture is therefore a significant source of error when analysing fresh silages and may also result in the overestimation of carbohydrate and protein degradability due to the incubation of a larger than predicted weight of DM.

7.5.2 COMPARISON OF THE ESTIMATED PROTEIN DEGRADATION PARAMETERS OF FRESH AND DRIED SILAGES

Using dried and milled material significantly underestimates the protein A fraction (P<0.05) and overestimates the protein B fraction (P<0.05) of fresh grass and maize silage (Table 7.8). This is assumed to be because oven drying of fresh material not only removes ammonia which is quickly degradable, but by difference also increases the concentration of non-volatile nitrogen fractions (Van Soest, 1982; Denium and Maasen, 1993) which are more likely to be slowly degraded and present in the protein B fraction. In agreement with the findings of other authors (Van Soest and Mason, 1991; Deinum and Maassen, 1994) this study also indicates that oven drying may alter the structure of the protein B fraction, which was shown to have a significantly lower rate of degradation in dried maize silage (P<0.01).

There was no significant effect (P>0.05) on the protein A or B fractions of wholecrop wheat after oven drying (Table 7.8) which may be related to the high initial DM of the fresh material (Table 7.7) which would be assumed to be correlated to a low concentration of QDP and may also have limited any further effect of oven drying on protein structure.

Assuming a passage rate of 0.08/h, this study showed that dried material underestimates the concentration of QDP available in fresh grass and maize silage and overestimated the concentration of ERDP. Although there was no significant difference in the concentration of DUP (P>0.05), the underestimation of QDP availability in the rumen when using dried material may result in a lower efficiency of feed protein utilisation by rumen microbes because of a provisional lack of quickly degradable carbohydrate in dairy rations. This highlights the importance of measuring the concentration of ammonia nitrogen in fresh silages to account for its loss during oven drying and its impact on the ruminal concentration of QDP.

Because this study showed no significant difference in the concentration of undegraded protein between dried and fresh silage samples, it is proposed that even though it may have a detrimental affect on the rate of protein
degradation, as previously stated, oven drying at 60°C does not significantly effect total protein degradability.

Although dried and milled material provides a homogenous sample the forage particles found in the rumen will be considerably larger than those found in the dried and milled material (Poppi et al., 1981). Drying and milling samples destroys the physical properties of the forage that determine its resistance to mastication and the surface area available for microbial attack (Hanna et al., 1973. It is these factors that determine the rate and extent of fermentation of forage protein and although dried and milled samples measure the maximum rate of protein degradation, without some measure of the physical characteristics of the fresh forage, dried material may over or underestimate the rate and extent of protein degradation in the rumen. (Nagadi et al., 1998; Lowman et al., 2002).

7.5.3 PREDICTING THE PROTEIN DEGRADATION PARAMETERS OF FRESH SILAGES USING DRIED MATERIAL

The developed multiple linear regression equations were only partially successful in predicting the protein degradation parameters of fresh forages from the protein degradation parameters of the same samples when dried and milled (Table 7.10). However, this investigation involved a limited number of samples of each forage type and further work needs to be conducted involving a larger number of samples to determine whether the kinetics of fresh silage protein degradation can be estimated from the incubation of dried and milled material.
There is no loss of soluble amino acids during oven drying, but there is a significant loss of moisture from fresh silages during processing to simulate mastication. Failure to correct for this results in the overestimation of the concentration of crude protein in fresh material.

Oven drying and milling of grass and maize silage results in the underestimation of the protein A fraction and concentration of QDP and the overestimation of the protein B fraction and the concentration of ERDP.
CHAPTER 8

8.1 GENERAL DISCUSSION

In recent years, the nutritional evaluation of feeds using in vitro systems has gained wider acceptance due to their repeatability and minimized use of fistulated animals. Amongst these in vitro systems, the in vitro gas production technique (IVGPT) has become increasingly popular due to its ability to provide kinetic data that describes the extent and rate of digestion from a single incubated sample.

The concept of using gas production as an index of feed degradation by rumen microbes has long been realised (McBee, 1953), but it was not until 1979 that a IVGPT was developed that allowed the kinetics of carbohydrate degradation to be routinely estimated (Menke et al., 1979). This technique involved measuring the cumulative gas produced by feeds incubated with buffered rumen liquor in air tight syringes and for the first time allowed feed degradation characteristics to be assessed on an industrial scale.

In principle, the only gas produced during IVGPT is as a result of microbial degradation of feed carbohydrate, either directly as carbon dioxide and methane or indirectly as carbon dioxide released when the short chain volatile fatty acids (VFA) produced during fermentation react with the bicarbonate buffer. Unfortunately, gas is also produced from a number of other sources (Steingass, 1983; Cone et al., 1997; Deaville and Givens, 1998; Cone and van Gelder, 1999), all of which must be accounted for before the carbohydrate degradation kinetics of the incubated sample can be assessed.

8.1.1 INTERFERENCE OF INDIRECT GAS PRODUCED BY SILAGE FERMENTATION ACIDS DURING IVGPT

During ensiling the anaerobic fermentation of plant organic matter produces VFA and lactic acid, which are crucial to preservation. In an attempt to avoid interference from indirect gas produced by these silage fermentation acids (SFA) reacting with the bicarbonate buffer all samples are routinely pre-dried before incubation to remove these SFA constituents (Deaville and Givens, 1998).
In this study, the indirect gas production attributed to SFA reached an asymptote at 30 minutes post incubation and although significantly higher in fresh silage material (80.9% higher (P<0.001) in fresh grass silage, 42.9% higher in fresh maize silage (P<0.05) and 25.5% higher in fresh wholecrop wheat (P<0.05)) there was still a significant volume of indirect gas produced during incubation of pre-dried material. These results indicate that although pre-drying removes a substantial component of SFA, which is assumed to be VFA, lactic acid and other non-volatile acids are not removed during drying and continue to contribute to indirect SFA gas production.

Failure to account for the indirect gas produced by SFA resulted in the overestimation of QCHO and TCHO and the underestimation of SCHO. The interference of SFA gas production was highest in grass silage (P<0.05), indicating that IVGP may overestimate the nutritive value of grass silage to a greater extent than that of maize silage or wholecrop wheat. Overestimating the quantity of fermentable carbohydrate in silages could result in the formulation of asynchronous diets that are not only deficient in total energy but also QCHO, resulting in the in the inefficient utilisation of QDP by rumen microbes and, in extreme cases, increasing the risk of ammonia toxicity and ketosis.

Based on the assumption that SFA are not utilised by rumen microbes (Deaville and Givens, 1998), correcting for the indirect gas produced by their reaction with the bicarbonate buffer should allow greater accuracy in prediction of carbohydrate availability in the rumen and a greater appreciation of the nutritive value of silages. To allow the routine quantification of indirect SFA gas production from silages during IVGP, equations were developed to predict the proportion of indirect SFA gas produced during the first 30 minutes of IVGP using proximate analysis values (DM, Lactic acid and VFA). Equations were successfully developed to predict the proportion of SFA attributable gas for fresh grass silage ($R^2 = 0.95$, $SEE = 0.05$, $P<0.001$), dried grass silage ($R^2 = 0.71$, $SEE = 0.13$, $P<0.05$), fresh wholecrop wheat ($R^2 = 0.59$, $SEE = 0.03$, $P<0.05$) and dried wholecrop wheat ($R^2 = 0.59$, $SEE = 0.02$, $P<0.05$). No successful regression equations were created to predict the proportion of indirect SFA gas produced by fresh or dried maize silage ($P>0.05$).
8.1.2 EFFECT OF SAMPLE PREPARATION AND PARTICLE SIZE ON THE CARBOHYDRATE DEGRADATION PARAMETERS OF SILAGE DURING IVGPT

Because fresh feeds are difficult to grind and often result in a heterogeneous sample of coarse particles (Lopez et al., 1995), it is common that in addition to being dried at 60°C for 24 hours, feeds are also milled through a 1mm sieve before incubation in IVGPT. This generates a homogenous sample that is easily stored but also destroys the physical characteristics of the feed and alters the chemical composition and structure of plant components (Pichard and Van Soest, 1977; Acosta and Kothmann, 1978; Van Soest, 1982; Piccaglia and Galleti, 1987; Chamley and Viera, 1990; Broesder et al., 1992).

Accurate assessment of the degradation characteristics of feeds is dependent on the incubated sample being representative of the feed as fed on farm. This may not be the case when analysing feeds after they have been dried and milled (Bonsi et al., 1995; Cone, 1998; Nagadi et al., 1998; Lowman et al., 2002) which in this study was shown to produce feed particles that were generally less than 0.5mm and therefore significantly smaller than those commonly found in the rumen (Dixon and Milligan, 1981; Dixon and Milligan, 1985; Cordoza and Mertens, 1986; Shaver et al., 1988; Grant et al., 1990; Beauchemin et al., 1994).

Smaller silage particles were shown to be fermented at a significantly higher rate with a higher concentration of QCHO (P<0.05) (Nagadi, 1999; Valentin et al., 1999) and a faster rate of fermentation of CHO (P<0.05), thus demonstrating the effect of particle size on fermentation kinetics. There was no significant difference in the TCHO concentration of the different particle sizes of grass silage (P>0.05), indicating that milling dried grass silage creates a homogenous sample with all parts of the plant represented in each particle fraction. In contrast, the concentration of SCHO and TCHO in maize silage and wholecrop wheat increased with decreasing particle size (P<0.05). This indicates that the different particle sizes of milled wholecrop cereals may represent different parts of the plant and because of the higher concentration of SCHO, the majority of small particles may represent grain starch. Depending on the correlation with grain breakdown during mastication and rumination, milling dried wholecrop cereals may result in the overestimation of starch availability using IVGPT.
Just as starch availability is an important component of SCHO, an important component of QCHO is the availability of soluble material such as simple sugars and short chain polymers (Stefanon et al., 1996). Because these are fermented significantly faster than the majority of insoluble material (Chesson and Forsberg, 1988; Stefanon et al., 1996) it is common to refer to all soluble material as being quickly degradable, even though there is some evidence to suggest that this is not always the case (Stefanon et al., 1996). This study showed that water soluble carbohydrate accounts for only 80.3% of the QCHO in silage and 19.7% of QCHO can be attributed to insoluble carbohydrates. A proportion of water soluble carbohydrate was also shown to be slowly fermented, with water soluble carbohydrate contributing 8.5% of the SCHO fraction. It is therefore inaccurate to describe soluble carbohydrates as QCHO and insoluble carbohydrates as SCHO.

Using IVGPT to compare the carbohydrate degradation parameters of fresh and dried silage material, fresh silage was shown to contain significantly more TCHO than dried material, principally related to a higher concentration of QCHO in fresh silage (P<0.05) and a higher concentration of SCHO in maize silage (P<0.05) and wholecrop wheat (P<0.05). There was no significant effect of drying and milling on the lag, but the rate of SCHO fermentation was 13.6% slower in fresh grass silage and 12.5% slower in fresh maize silage compared to dried material. These results indicate that the use of dried material may underestimate the nutritive value of silage but overestimate the rate of fermentation because of the smaller particle size of the incubated sample.

Because fresh feeds are difficult handle, equations were developed to predict the gas production parameters of fresh silage when incubating dried and milled silage material during IVGPT. Assuming the rate of fermentation of QCHO is 0.2 h⁻¹ (see Appendix A.2 for details) the gas production data of dried material can be used to predict the volume of gas produced by QCHO (A) in fresh grass silage (R²=90.4, P<0.001), fresh maize silage (R²=81.3, P<0.001) and fresh wholecrop wheat (R²=76.5, P<0.001) as well as the volume of gas produced by SCHO (B) (R²=81.5, P<0.01; R²=73.3, P<0.01; R²=84.4, P<0.01), the respective lag (R²=81.3, P<0.001; R²=82.1, P<0.001; R²=83.8, P<0.01) and rate of SCHO fermentation (C₅) (R²=79.0, P<0.01; R²=95.1, P<0.001; R²=94.2, P<0.001).
8.1.3 EFFECT OF AMMONIA AVAILABILITY ON THE DEGRADATION PROFILE OF CARBOHYDRATES DURING IVGPT

As well as being dependent on carbohydrate availability, cumulative gas production is also sensitive to nitrogen availability (Raab et al., 1983; Nagadi., 1999). This is because the rate of microbial fermentation is highly dependent on the availability of energy and protein which are required for maintenance and microbial protein synthesis (MPS). Consequently, to ensure that the rate of gas production during IVGPT is directly related to the availability of fermentable carbohydrate, feed samples are usually incubated in a nitrogen rich environment (Hungate, 1966; Erdman et al., 1986).

The microbial requirement for nitrogen to support maintenance and growth requirements means that as the availability of fermentable carbohydrate increases so too does the requirement for nitrogen (Raab et al., 1983; Nagadi, 1999). This was observed in this study where it was shown that the positive relationship between gas production and the quantity of incubated carbohydrate (sucrose: \( R^2 = 98.7 \ P < 0.001 \); maize starch: \( R^2 = 99.5 \ P < 0.001 \); and glucose: \( R^2 = 98.7 \ P < 0.001 \) was also associated with a negative linear relationship with the final nitrogen level (sucrose: \( R^2 = 98.3 \ P < 0.001 \); maize starch: \( R^2 = 99.9 \ P < 0.001 \)).

When nitrogen availability limits MPS a larger proportion of the available carbohydrate is used for microbial maintenance and the volume and rate of gas production declines (Nagadi, 1999). The sensitivity of IVGPT to nitrogen availability was further demonstrated by Krishnamoorthy et al., (1991) who showed that during incubation of a carbohydrate mixture there is a linear relationship between MPS and cumulative gas production in the first 8 hours. After 8 hours, Russel et al., (1992) showed that MPS and gas production is dependent on both the rate of carbohydrate fermentation and nitrogen availability. This was also observed in this study which showed that although the volume of gas produced during IVGPT was dependent on nitrogen availability, the volume and rate of gas production was also dependent on the quantity and type of carbohydrate substrate.

There was no significant difference in the efficiency of nitrogen utilisation between different carbohydrate sources (\( P > 0.05 \)) but the volume and rate of gas production in the first 24 hours was significantly lower when incubating maize starch compared to sucrose or glucose (\( P < 0.05 \)). Because rumen microbes were shown to utilise sucrose and glucose with the highest level of
efficiency (P<0.05) it is proposed that these sugars promotes a higher level of MPS compared to maize starch.

The efficiency of carbohydrate utilisation by the rumen microbes was positively correlated to nitrogen and carbohydrate inclusion (P<0.05), but only up to 100mg of carbohydrate and to an ammonia concentration of 78.4mgN/l. As the ammonia concentration increased from 78.4mgN/l to 102.1 mgN/l there was no significant further increase in the efficiency of carbohydrate utilisation by the rumen microbes. This negative effect of high ammonia concentration (102mgN/l) on the efficiency of carbohydrate utilisation was partially negated by increasing the quantity of incubated carbohydrate (100mg vs. 150mg) but the efficiency of carbohydrate utilisation was never significantly higher than that achieved at 78.4mgN/l (P>0.05). This indicates that although there is a beneficial effect of ammonia supplementation of gas production during IVGPT as shown by Nagadi (1999), elevated concentrations of rumen ammonia can have a detrimental effect on microbial fermentation and MPS by causing an increase in maintenance energy demand (Lay et al., 1998; Sung and Lui, 2003). Based on this observation it is suggested that to maximise the rate of carbohydrate fermentation and MPS the optimum rumen ammonia concentration should be 78.4mgN/l. This is significantly higher than proposed by Satter and Slyter (1974) (50 mgN/l) but similar to that proposed by Hume et al., (1970) (88 mgN/l) and Hoover (1986) (80 mgN/l).

IVGPT is therefore most sensitive to nitrogen availability when the incubated carbohydrate to nitrogen ratio is 42:1 with a maximum nitrogen concentration of 78.4mgN/l. At these levels of inclusion the efficiency of microbial nitrogen utilisation is maximised and gas production is positively correlated to nitrogen availability. This sensitivity of IVGPT to nitrogen availability also means that it could be used as an index of MPS and nitrogen availability to measure the protein degradation characteristics of feeds when incubated in nitrogen free rumen fluid.

8.1.4 ABSORBANCE OF AMMONIUM BY ZEOLITE

Adapting IVGPT to measure the kinetics of protein degradation therefore requires the use of nitrogen free bicarbonate buffer and rumen fluid with all background nitrogen removed.

Centrifugation has previously been used to reduce the ammonia concentration of rumen fluid with no apparent detrimental effect on
microbial activity (Nagadi, 1999). However, centrifugation is a time consuming process and results in the rumen microbes being aerobic and below 39°C for a considerable period of time. An alternative is the rapid filtration of rumen fluid through zeolite, which is an aluminosilicate mineral used for decades to remove ammonium from effluent (Mercer et al., 1970) and ammonia from waste water (Besser et al., 1998).

The ammonium absorption capacity of zeolite during column filtration is significantly affected by the bed volume flow rate (BV/h) (Hlavay, et al., 1982; Booker et al., 1996), with absorption significantly decreasing with increasing flow rate up to 68.2 BV/h (P<0.001). This effect of flow rate on ammonium absorption was shown to be best described using a 3-phase hyperbolic decay curve (R²=0.998, P<0.001), which showed that 2-3mm zeolite has a minimum absorption coefficient of ~1mgNH₃-N/g zeolite. However, to ensure sufficient absorption from rumen liquor with an unknown ammonium concentration the most economical flow rate suggested by this study was 8.93 BV/h. Although higher than the suggested optimal flow rate (5-7 BV/h) (Hlavay, et al., 1982; Booker et al., 1996) it is within the target range for column filtration (Booker et al., 1996) and equates to an absorption coefficient using 2-3mm zeolite of 2.95 mgNH₃-N/g.

Filtering rumen fluid through zeolite had no significant effect on the size of the microbial population estimated from its absorbance at 600nm (P>0.05) but significantly increased microbial performance when fermenting sucrose during IVGPT. Sucrose incubated in filtered rumen fluid produced 2.9% more gas in the first 6 hours (P<0.05), had a shorter lag (P<0.001) and produced 1.7% more total gas (P<0.01) than when incubated in unfiltered rumen liquor. This superior microbial activity may be due to selective retention of protozoa in the zeolite column. Protozoa are considerably larger than the rumen bacteria upon which they usually predate (1-1000μm vs. 0.64-1.5μm) and if retained in the column would result in a larger active bacterial population. If this is true the improvement in microbial activity would be in agreement with other authors who have demonstrated the positive effects of in vivo defaunation on the microbial growth (Eugène et al., 2004), animal performance and feed conversion efficiency (Santra and Karim, 2000; Eugène et al., 2004).
8.1.5 COMPARISON OF GRASS SILAGE PROTEIN DEGRADATION ESTIMATED USING THE NYLON BAG TECHNIQUE AND IVGPT

Adapting IVGPT to measure the protein degradation kinetics of feeds may significantly improve the description of protein availability in the rumen, which is currently assessed using the nylon bag technique (Ørskov et al., 1980).

In addition to being tedious and expensive, the nylon bag technique assumes that all feed protein lost from the nylon bag is quickly degraded, particularly the water soluble fraction which is assumed to be degraded at an infinite rate (AFRC, 1988). In contrast, IVGPT measures the products of protein fermentation rather than its assumed degradation via loss from the nylon bag and therefore proves kinetic data describing the extent and rate of digestion of all the protein fractions, including QCHO. The disadvantage of IVGPT is that there are no effects of passage rate as in the nylon bag technique, meaning that the availability of protein in the rumen can only be assessed using a dynamic rumen model and incorporating a measure of soluble feed protein concentration.

Soluble feed protein is assessed (Steen et al., 1998; Dawson and Steen, 2000) as the proportion of nitrogen washed out of ANKOM fibre bags with a pore size of 20-25µm. Because the bags used in the nylon bag technique have a significantly larger pore size (1600 µm) it was shown that 18.7% of the water soluble nitrogen fraction measured could be attributed to insoluble fine feed particles. Consequently, the nylon bag technique over predicts the concentration of soluble nitrogen in grass silage by 23%, with no significant correlation shown between soluble nitrogen fraction measured using the nylon bag technique and the standard measure of protein solubility using ANKOM fibre bags (P>0.05).

The IVGPT estimate of the protein A fraction of grass silage was 40.2% lower (P<0.001) than that estimated using the nylon bag technique and was significantly correlated to the concentration of soluble non-ammonia nitrogen (SNAN) (R²=77.9, P<0.001). These results further indicated that only 76.3% of SNAN in grass silage is actually quickly degraded and up to 37.5% may be slowly degradable. This conclusion is supported by other authors who have shown that soluble protein may not be immediately degraded and may in fact remain undegraded in the rumen for 8-12 hours (Fahmy et al., 1991; Aufrère et al., 1994). Consequently, soluble protein in the liquid phase
of rumen passage may make a significant contribution to the by-pass protein fraction of feeds with (Aufrère et al., 2001).

The concentration of quickly degradable protein (QDP) estimated using IVGPT was 52.7% less (P<0.001) than that estimated using the nylon bag technique, which also estimated a significantly smaller concentration of slowly degradable protein (SDP) (P<0.001). Although there was no significant difference in the rate of degradation of SDP estimated by both techniques, assuming a passage rate of 0.08/h, the estimated concentration of effective rumen degradable protein (ERDP) was 16.7% lower (P<0.001) using IVGPT. Because there was also no significant difference in the concentration of undegraded protein estimated by each technique it would be expected that the IVGPT estimated concentration of digestible undegraded protein (DUP) would be 16.7% higher than that estimated using the nylon bag technique. In reality the IVGPT estimate of DUP is 155.7% higher (P<0.001). This is because whilst assuming that only 80% of QDP is utilised by rumen microbes, the nylon bag technique also assumes that no QDP escapes rumen fermentation, resulting in 20% of the QDP fraction being unaccounted for. Because in vivo experiments have shown that QDP does contribute to the DUP fraction (Aufrère et al., 2001; Choi et al., 2002; Volden et al., 2002) the results of this study indicate that the nylon bag technique underestimate the concentration of DUP supplied by a diet and may result in the overfeeding of supplementary dietary protein.

8.1.6 EFFECT OF SAMPLE PREPARATION ON THE PROTEIN DEGRADATION PARAMETERS OF GRASS SILAGE, MAIZE SILAGE AND WHOLECROP WHEAT ESTIMATED USING IVGPT

Whilst early studies have demonstrated that drying and milling has a significant effect on the degradation profile of silage carbohydrates (see Chapter 3), these effects could be even greater on silage protein which is especially sensitive to the method of preservation (Abdalla et al., 1988).

Fresh silages were estimated to have significantly higher concentration of crude protein (CP) compared to the dried material (P<0.001). It was assumed that this was due to the loss of ammonia during oven drying, but when the contribution of ammonia was removed the CP concentration was still 20.5% higher in fresh grass silage (P<0.001), 11.4% higher in fresh maize silage (P<0.01) and 31.2% higher in fresh wholecrop wheat (P<0.001). These results
were unexpected and indicated that there was either an additional source of protein loss and/or an error in the analytical procedure.

To establish whether soluble aromatic and heterocyclic free amino acids could also be lost during oven drying, solutions of L-Proline, L-Alanine and L-lysine where placed in a 60°C oven. After 24 hours it was shown that there was no loss of amino acid nitrogen from these solutions, indicating that soluble free amino acids are not lost during oven drying and did not account for the additional protein loss.

Accurate assessment of the CP concentration of fresh sample is reliant on an accurate estimate of the DM of the analysed sample. Further investigation showed that there was a significant loss of moisture during processing of fresh material in the electric blender. Failure to correct for this additional loss of moisture results in the overestimation of CP concentration and may also result in the overestimation of carbohydrate and protein degradability, due to a larger weight of DM being incubated.

After correcting for moisture loss, this study showed that dried and milled material significantly underestimated the protein A fraction (P<0.05) and significantly overestimated the protein B fraction (P<0.05) of fresh grass silage and fresh maize silage estimated during IVGPT. These results show that oven drying not only removes ammonia, which will be an important component of the protein A fraction, but also increases the concentration of non-volatile nitrogen fractions (Van Soest, 1982; Denium and Maasen, 1993) which are more likely to be slowly degraded and present in the protein B fraction. There was no significant difference in the protein degradation parameters of dried and fresh wholecrop wheat (P>0.05) which may be due to the fact that the wholecrop wheat samples were significantly drier than the grass and maize silage samples and consequently the effects of oven drying may have had a limited effect on any further ammonia loss.

There was no significant difference in the observed lag or rate of degradation of the protein B fraction between dried and fresh grass silage or wholecrop wheat (P>0.05), but the protein B fraction of fresh maize silage was shown to be degraded at significantly higher rate than that in dried material (P<0.01). In agreement with the findings of other authors (Van Soest and Mason, 1991; Deinum and Maassen, 1994) these results indicate that oven drying may alter the structure of the protein B fraction, particularly in maize silage.
The use of oven dried and milled material was shown to underestimate the concentration of QDP and overestimate the concentration of ERDP available in fresh grass silage and fresh maize silage. Although there was no significant difference in the estimate concentration of DUP between fresh and dried material (P>0.05), the under estimation of QDP using dried material could result in diets being formulated with a provisional lack of QCHO, resulting in a lower efficiency of protein utilisation by rumen microbes.

However because fresh silages are inherently difficult to handle, equations were developed in an attempt to predict the protein degradation parameters of fresh silages when incubating dried and milled silage material during IVGPT. Based on the assumption that the rate of QDP degradation is 0.3 h⁻¹, these equations were only partially successful but made it possible to predict the proportion of quick A protein in fresh grass silage (R²=0.958, P<0.001), the proportion of slow B protein in fresh grass silage (R²=0.958, P<0.001), maize silage (R²=0.93, P<0.001) and wholecrop (R²=0.985, P<0.001) and the rate of slow B protein degradation (Cs) in fresh maize silage (R²=0.99, P<0.001) and wholecrop (R²=0.98, P<0.01) using dried and milled silage protein parameters.
8.2 GENERAL CONCLUSIONS

- SFA produce indirect gas in the first 30 minutes of IVGPT which has a significant effect on the gas production profile and subsequent degradation parameter estimates of both dried and fresh silages.
- There is no significant difference in the mean particle size of dried silages milled to pass through a 1mm sieve but different particle sizes represent different parts of the plant, with the majority of small wholecrop cereal particles represented by grain.
- Not all water soluble carbohydrate is quickly fermented by rumen microbes and not all water insoluble carbohydrates are slowly fermented.
- Fresh silages contain significantly more TCHO than dried material with a higher concentration of QCHO in fresh grass silage and a higher concentration of SCHO in maize silage and wholecrop wheat. There is no effect on lag, but SCHO is fermented significantly slower in fresh grass and maize silage compared to dried material.
- Rumen ammonia levels greater than 78.4 mgN/L have a detrimental effect on the efficiency of microbial digestion and growth.
- IVGPT is sensitive to nitrogen availability and is most sensitive when the incubated sucrose to nitrogen ratio is 42:1.
- Filtering rumen liquor through zeolite at 8.93 BV/h removes ammonium with a coefficient of 2.95mgNH₄-N/g with no detrimental effect on microbial activity.
- Due to the loss of fine insoluble feed particles, the nylon bag technique overestimates the concentration of soluble nitrogen by 23%.
- The protein A fraction measured using IVGPT is significantly correlated to soluble NAN content and indicates that only 76.3% of soluble NAN in grass silage is quickly degraded.
- IVGPT estimates grass silage to have a significantly lower concentration of QDP and ERDP and a significantly higher concentration of SDP and DUP than estimated using the nylon bag technique.
- There is no loss of soluble amino acids during oven drying, but there is a significant loss of moisture from fresh silages during simulated mastication.
- The oven drying and milling of grass and maize silage results in the underestimation of the protein A fraction and QDP and the overestimation of the protein B fraction and ERDP.
8.2.1 RECOMMENDED METHODOLOGY FOR THE ESTIMATION OF SILAGE PROTEIN DEGRADATION USING IVGPT

1. Prepare stock solutions
   - Main element solution: 5.7g Na₂HPO₄ + 6.2g KHPO₄ + 0.6g MgSO₄·7H₂O in 1 litre of distilled water
   - Buffer solution: 39g NaHCO₃ in 1 litre of distilled water
   - Trace element solution: 13.2g CaCl₂·2H₂O + 10g MnCl₂·4H₂O) + 1g CoCl₂·6H₂O + 0.89 FeCl₂·6H₂O) in 100ml of distilled water
   - Resazurin solution: 100mg resazurin in 100ml of distilled water
   - Reduction solution: Prepared on day.

2. Wash zeolite by firstly sieving through a 500μm sieve and then repeatedly wash in a clean bucket with distilled water. Transfer zeolite to a 60°C oven overnight and then repeat the procedure a second time before finally storing the dried and washed zeolite in a clean plastic bag.

3. Measurement of microbial activity of rumen liquor

   Strain rumen liquor through two layers of 250 μm netting and three layers of muslin, mixed and then diluted with main element solution:

   - 1ml liquor + 2ml of main element solution
   - 1ml liquor + 4ml of main element solution
   - 1ml liquor + 8ml of main element solution

   Further dilute 200μl of each dilution volumetrically to 10ml using main element solution and measure its absorbance at 600nm using a spectrophotometer. Select the dilution ratio that has an absorbance of 0.111-0.134.

4. Filter the rumen liquor

   Place approximately 200g of washed and dried zeolite into two glass columns and wash with distilled water until filtrate is clear. Connect each column to a 500ml aspirator bottle situated above each column in a thermos box, and suspended over a 39°C water bath containing two 500ml bottles. Pour 500ml of rumen liquor into each column and set
the column flow rate at 8.92 BV/h. Flush the filtered rumen liquor with CO₂ and store in a thermos flask.

5. Prepare Buffer media

Prepare the correct volume of buffer and reduction solutions using the appropriate dilution ratios and referring to Table A.1. Incubate both solutions in a 39°C water bath, flush the buffer solution with CO₂ and mix with the reduction solution when solution becomes colourless. Add the correct volume of filtered rumen liquor and gas with CO₂ for 5 minutes.

6. Prepare syringes

Weigh 100mg of sucrose into a 100ml glass syringe fitted with silicone rubber tubing and a plastic clip. According to the following equation weigh out the calculated amount of each sample to be analysed in triplicate into the appropriate glass syringe:

\[
\text{Sample (mg)} = \left(\frac{1000}{\text{CP (g/kgDM)}}\right) \times 0.1875 \text{ mgCP/30ml} \times 78.4 \text{ mgN/L}
\]

Prepare three blank syringes containing 100mg of sucrose but no sample. Lightly lubricate the plungers with Vaseline, insert into the syringes, close the clip and suspend from a Perspex lid into a 39°C water bath for 1 hour.

7. Inoculate and start IVGPT

Transfer 30ml of inoculum into each syringe using a automatic dispensing pump. Close the clip, read and record initial volume and time before shaking and returning the syringe into the water bath.

Record the gas produced every very half hour for the first 3 hours, hourly until 6 hours and thereafter at 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 56, 64, and 72h. Manually shake the syringe after each reading and re-calibrate the syringes if the gas volume exceeds 50ml.

8. Measure undegraded residual nitrogen

After 72 hours empty each syringe into a 200ml medicinal bottle, mix with 15ml of NDF solution and autoclave at 105°C for 1 hour. Filter
the contents of each bottle through Whatman 54 Hardened filter paper (20-25μm) using a buchner funnel and rinse with distilled water and alcohol. Dry the filter paper in a 60°C oven, roll and cut into digestion tubes and then digest for 1 hour with 20ml of concentrated H₂SO₄ and 3 catalyst tablets. Distil according to Kjedhal using a Buchii Distillation unit and then titrate with 25M H₂SO₄ in aliquots of 0.1ml.

Undegraded Nitrogen (mgN) = Sample residue plus filter paper (mgN) – Blank syringe residue plus filter paper (mgN)

9. Analyse the gas production data

Transform the cumulative gas production:

- Correct for the appropriate weight of sucrose and sample
- Correct for blank gas production by fitting the model: Gas = A(1-exp(-cᵢt))+B(1-exp(-cₛ(t-lag)))*exp(5(t-l))/\left(1+exp(5(t-lag))\right) to the mean gas production of the blank syringes and subtract from all the sample gas profiles.
- Correct for indirect silage SFA gas production
- Modify the sample gas profiles by applying the equation:

Modified volume at y (ml) = (Adjusted volume at y (ml) * ((Blank volume at y (ml) – Blank volume at x (ml)) / Blank volume at y (ml))) + Modified volume at x (ml)

Where x and y represent sequential time points
- Modify the gas volumes to represent units of degraded protein:

Proportion of CP at 72h n (%CP) = (Modified volume at n (ml)) * ((2.37 mgN – undegraded nitrogen (mgN)) / 2.37 mgN)

Were n represents time (n-th hour)
- Describe the protein parameters by fitting the equation Gas = A(1-exp(-cᵢt))+B(1-exp(-cₛ(t-lag)))*exp(5(t-l))/\left(1+exp(5(t-lag))\right) where A is the proportion of QDP, ‘Cᵢ’ is the fractional rate of degradation of QDP (fixed at > 0.3/h, ‘B’ is the proportion of SDP, ‘Cₛ’ is the fractional rate of degradation of SDP (/h), ‘t’ is time (h) and ‘lag’ is the discrete time lag (h) before the fermentation of SDP begins.
Work on this thesis has resulted in the following publication:


Available at:

http://authors.elsevier.com/sd/article/S037784010500194X
References


Bioparametrics Ltd, Peter Wilson Building, West Mains Road, Edinburgh, EH9 3JG. www.bioparametrics.com


Bonsi, M.C.K., Osuji, P.O., Tuah, A.K., 1995. Effect of supplementing Teff straw, with different levels of leucaena or sesbania leaves on the degradabilities of teff straw, sesbania, leucaena, tagasate and veronia and on certain rumen and blood metabolites in Ethiopian Menz sheep. Animal Feed Science and Technology 52, 101-129.


Forage Analysis Assurance (FAA) group, Highfield, Little London, Andover, UK


McBee, R.H., 1953. Manometric method for the evaluation of microbial activity of rumen fluid with application to utilisation of cellulose and hemicellulose. Applied Microbiology 1, 106-110


Zhu, W-Y., Kingston-Smith, A.H., Troncoso, D., Merry., R.J., Davies, D.R., Pichard, G., Thomas, H., Theodorou, M.K., 1999. Evidence of a role for plant proteases in the

A.1 General Material and Methods

A.1.1 The preparation of forage samples

All dried forages used in this thesis were dried for 24 hours at 60°C and then milled using a Retsch® mill through a 1mm screen. The simulated mastication of fresh samples used in this thesis was achieved by freezing the samples overnight and then chopping approximately 15g of the frozen sample at 1450rpm for 15 seconds in a Moulinex Ovatio 3 Duo blender.

A.1.2 Proximate analysis methods for forage samples

Neutral Detergent Fibre (NDF)

NDF was determined according to the method of Van Soest et al., (1991) using ANKOM F57© fibre bags with a pore size of 20-25μm. The method is defined below:

The fibre bags were labelled and weighed (W1) and then approximately 500mg of dried material was weighed into each bag (W2). Each bag was sealed using a heat sealer and then transferred into a round bottom flask containing 1 L of NDF solution (20 fibre bags /L NDF). The flask was then left to simmer in an electromantle (Electrothermal, Southend, UK) for 1 hour with intermittent stirring. After 1 hour the NDF was poured off and the bags covered with approximately 500ml of enzyme solution, placed on a magnetic stirrer hot plate and stirred for 15 minutes. After 15 minutes the enzyme solution was poured off and replaced with another 500 ml of enzyme solution which was left to stir for another 15 minutes. The enzyme solution was then poured off and the fibre bags washed with boiling distilled water until there was no further colour change in the water. The fibre bags were then dried for 24 hours at 60°C and then weighed (W3).

\[
\text{NDF (g/kgDM)} = \frac{[W_3 - (W_1 \times C_i)] \times 1000}{W_2}
\]

(Blank correction value \(C_i\) = Final oven dried weight / Original blank bag weight)
*Acid Detergent Fibre (ADF)*

ADF was determined using ANKOM F57© fibre bags with a pore size of 20-25μm. The method is defined below:

The fibre bags were labelled and weighed \(W_1\) and then approximately 500mg of dried material was weighed into each bag \(W_2\). Each bag was sealed using a heat sealer and then transferred into a round bottom flask containing 1 L of ADF solution (20 fibre bags /L NDF). The flask was then left to simmer in an electromantle (Electrothermal, Southend, UK) for 1 hour with intermittent stirring. After 1 hour the ADF solution was poured off and the bags washed with boiling distilled water until there was no further colour change in the water. The fibre bags were then dried for 24 hours at 60°C and then weighed \(W_3\).

\[
ADF \text{(g/kgDM)} = \frac{(W_3 - (W_1 \times C_1)) \times 1000}{W_2}
\]

(Blank correction value \(C_1\) = Final oven dried weight / Original blank bag weight)

*Crude Protein (CP)*

The crude protein content of the forage samples was determined using the Kjeldhal method incorporating a Buschi Distillation Unit.

**A.1.3 Ammonia concentration measurements**

The ammonia concentration of the standard ammonia solutions was measured using a FOSS FIAstar 5000 Analyzer fitted with a 5027 sampler unit (Chapter 6). The ammonia level of rumen liquor was measured using an ammonia probe (Chapter 3).

**A.1.4 Carbohydrate in vitro gas production technique**

1. *Preparation of the solutions*

The stock solutions were made using the following procedure:

- Main element solution : 5.7g Na₂HPO₄ + 6.2g KHPO₄ +0.6g MgSO₄.7H₂O in 1 litre of distilled water
• Buffer solution: 35g NaHCO₃ + 4g (NH₄)HCO₃ in 1 litre of distilled water
• Trace element solution: 13.2g CaCl₂.2H₂O + 10g MnCl₂.4H₂O + 1g CoCl₂.6H₂O + 0.89 FeCl₂.6H₂O in 100ml of distilled water
• Resazurin solution: 100mg resazurin in 100ml of distilled water
• Reduction solution: Prepared on day.

With the exception of the reduction solution, all these stock solutions were prepared and stored a short time prior to incubation.

8. Measurement of microbial activity in rumen liquor

The rumen liquor was strained through two layers of 250 μm netting and three layers of muslin, mixed and then diluted with main element solution:

• 1ml liquor + 2ml of main element solution
• 1ml liquor + 4ml of main element solution
• 1ml liquor + 8ml of main element solution

200μl of each dilution was then further dilute to 10ml volumetrically with main element solution. The spectrophotometer was set to 600nm and calibrated against main element solution. The absorbance of each dilution was measured at 600nm and the dilution ratio selected which gave an absorbance reading in the range of 0.111-0.134.

9. Preparation of Buffer media

Having selected the appropriate dilution ratio the correct volumes of buffer medium and reduction solution were prepared (see Table A.1) and incubated in a water bath at 39°C. Carbon dioxide was flushed through the buffer medium and then the reduction solution was added. When the mixed buffer media had become colourless, the correct volume of rumen liquor was added and the inoculum gassed with carbon dioxide for a minimum of 5 minutes.

10. Procedure

Approximately 220mg of each sample was weighed into a 100ml glass syringe (Haberle, Labortchnik) fitted with a length of silicone rubber tubing and plastic clip. Three blanks syringes were also prepared which contained no sample. The plungers were lightly lubricated with Vaseline to create an air tight seal and ease movement, inserted into the syringe and the clip
closed. The syringes were suspended from a Perspex lid and pre-warmed in a 39°C water bath for 1 hour before 30 ml of the inoculum was transferred using an automatic dispenser (Jencons perimatic GP) into each syringe through the rubber tubing. The plastic clip was closed, the position of the plunger read and the initial volume and time recorded. The syringe was then likely shaken and placed back into the water bath.

Gas production was recorded and the syringes manually shaken every half hour for the first 3 hours, then hourly until 6 hours and thereafter at 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 56, 64, and 72h according to Jessop and Herrero (1996).

If the gas volume exceeded 50ml, the reading was recorded, the syringe inverted, the plastic clip opened and the plunger gently returned to the 30ml mark. The new volume was recorded and the incubation allowed to continue.

5. Analysing the gas production data

The cumulative gas production was analysed using a non-linear procedure by fitting the model: $\text{Gas} = A(1-\exp(-c\cdot t))+B(1-\exp(-c\cdot(t\text{-lag})))^e\exp(5(t-\text{l}))/(1+\exp(5(t-\text{l})))$ modified from Jessop and Herrero (1996). The original model incorporated a $t > 1$ function which was incompatible with the SigmaPlot© (2002) curve fitting software used. Thus the model was modified and the $t > 1$ function replaced with a steep growth curve of $(\exp(5(t-\text{l}))/1+\exp(5(t\text{-lag})))$ (Green, 2002). This model was chosen because of the biological relevance of its parameters. $A$ is the asymptotic gas production (ml) from fermentation of quickly degraded carbohydrate (QCHO), ‘$C_r$’ is the fractional rate of gas production of $A$ (fixed at $> 0.2$/h), ‘$B$’ is the asymptotic gas production (ml) from fermentation of slowly degraded carbohydrate (SCHO), ‘$C_s$’ is the fractional rate of gas production of $B$ (/h), ‘$t$’ is time (h) and ‘lag’ is the discrete time lag (h) before the fermentation of SCHO begins.
Table A.1 Rumen inoculum preparation for the in vitro gas production technique based on a dilution ratio of 1:2 v/v

<table>
<thead>
<tr>
<th>Medium: Liquor (1:2 v/v)</th>
<th>No. of Syringes</th>
<th>Buffer Medium</th>
<th>Reduction Soln.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Dist.water cm³</td>
<td>Trace elemt. cm³</td>
</tr>
<tr>
<td>20:10</td>
<td>1</td>
<td>10</td>
<td>0.003</td>
</tr>
<tr>
<td>200:100</td>
<td>10</td>
<td>100</td>
<td>0.03</td>
</tr>
<tr>
<td>300:150</td>
<td>15</td>
<td>150</td>
<td>0.04</td>
</tr>
<tr>
<td>400:200</td>
<td>20</td>
<td>200</td>
<td>0.05</td>
</tr>
<tr>
<td>600:300</td>
<td>30</td>
<td>300</td>
<td>0.08</td>
</tr>
<tr>
<td>800:400</td>
<td>40</td>
<td>400</td>
<td>0.1</td>
</tr>
<tr>
<td>1000:500</td>
<td>50</td>
<td>500</td>
<td>0.13</td>
</tr>
<tr>
<td>1200:600</td>
<td>60</td>
<td>600</td>
<td>0.15</td>
</tr>
<tr>
<td>1400:700</td>
<td>70</td>
<td>700</td>
<td>0.18</td>
</tr>
<tr>
<td>1600:800</td>
<td>80</td>
<td>800</td>
<td>0.2</td>
</tr>
<tr>
<td>2000:1000</td>
<td>100</td>
<td>1000</td>
<td>0.3</td>
</tr>
</tbody>
</table>

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The cumulative gas volumes were first corrected to fermentation of 220mgDM and then corrected for the fermentation of endogenous liquor material and microbial turnover by fitting the gas production profiles of the ‘blanks’ to the modified model of Jessop and Herrero (1996). Assuming a microbial lag of 24 h, the mean cumulative gas production of the blanks was then subtracted from all gas profiles. The adjusted cumulative gas volumes for each sample were then corrected for silage fermentation acid (SFA) gas production as described in Chapter 1, before finally being fitted to the modified model of Jessop and Herrero (1996) to estimate the degradation parameters.

To represent the cumulative gas production in terms of the quantity of carbohydrate degraded (g/kgDM), the degradation parameters were transformed. The A fraction was converted to QCHO (g/kgDM) by firstly correcting for the gas produced by protein fermentation, which is assumed to mainly occur in the A fraction (Cone and Van Gelder, 1999), by using a multiplication factor of 0.16 as proposed by Jessop (2004) and then applying a multiplication factor of 0.36 which is based on the gas production of glucose (Jessop and Herrero, 1996). The B fraction was converted to SCHO (g/kgDM) by applying a multiplication factor of 0.47 which is based on the gas production of NDF (Herrero and Jessop, 1996):

\[
QCHO \text{ (g/kg DM)} = \frac{((A(ml)-(A(ml)*0.16))*1000)}{\text{sample weight (mg))}/0.36
\]

\[
SCHO \text{ (g/kg DM)} = \frac{(B(ml)*1000)/\text{sample weight (mg))}}{0.47}
\]

A.1.5 Protein in vitro gas production technique

1. Preparation of the solutions

The stock solutions were made using the following procedure:

- Main element solution : 5.7g Na₂HPO₄ + 6.2g KHPO₄ +0.6g MgSO₄·7H₂O in 1 litre of distilled water
- Buffer solution : 39g NaHCO₃ in 1 litre of distilled water
- Trace element solution: 13.2g CaCl₂·2H₂O + 10g MnCl₂·4H₂O +1g CoCl₂·6H₂O + 0.89 FeCl₂·6H₂O) in 100ml of distilled water
- Resazurin solution: 100mg resazurin in 100ml of distilled water
- Reduction solution: Prepared on day.
2. **Washing of zeolite**

Approximately 2 kg of the raw zeolite was sieved through a 500µm sieve to remove very small particles and then transferred to a clean bucket where it was repeatedly washed with distilled water. After approximately eight washes the zeolite was transferred into three large foil dishes and placed in a 60°C oven for 12 hours. The dried zeolite was then sieved for a second time through a 500µm sieve, washed with distilled water approximately 5 times and then dried in a 60°C oven for 12 hours. The dried zeolite was then sieved through a 500µm sieve for the final time and stored in a sealed plastic bag.

3. **Measurement of microbial activity of rumen liquor**

The rumen liquor was strained through two layers of 250 µm netting and three layers of muslin, mixed and then diluted with main element solution:

- 1ml liquor + 2ml of main element solution
- 1ml liquor + 4ml of main element solution
- 1ml liquor + 8ml of main element solution

200µl of each dilution was then further dilute to 10ml volumetrically with main element solution. The spectrophotometer was set to 600nm and calibrated against main element solution. The absorbance of each dilution was measured at 600nm and the dilution ratio selected which gave an absorbance reading in the range of 0.111-0.134.

4. **Filtering the rumen liquor**

Approximately 200g of washed and dried zeolite was poured into two glass columns (length 300mm, radius 17.5mm) fitted with a section of rubber tubing and a variable plastic clamp. The glass columns were held in a vertical position under 500ml aspirator bottles contained in a thermos box and connected to the columns with rubber tubing and through a rubber stopper. The glass columns were fed into 500ml bottles placed in a 39°C water bath underneath the columns.

With the clamp open the zeolite was washed three times with distilled water to remove any fine particles, to saturate the zeolite and reduce absorption of the rumen liquor. The effect of each washing and the clarity of the water
collected was assessed by placing the beaker over a black cross drawn on a white background.

After the zeolite was washed, the clamp was closed and the glass columns connected to the 500ml bottles contained in the thermos box. 500ml of rumen liquor was poured into each 500ml aspirator bottle using a funnel, the thermos lid replaced and the rumen liquor allowed to drain into each column. When the column was full, the clamp was opened sufficiently wide to produce a flow rate of 8.92 BV/h. After filtration, the rumen liquor was mixed and stored in a thermos flask gassed with CO₂.

11. Preparation of Buffer media

Having selected the appropriate dilution ratio the correct volumes of buffer medium and reduction solution were prepared (see Table A.1) and incubated in a water bath at 39°C. Carbon dioxide was flushed through the buffer medium and then the reduction solution was added. When the colour of the mixed buffer media had become colourless, the correct volume of rumen liquor was added and the inoculum gassed with carbon dioxide for a minimum of 5 minutes.

12. Procedure

Approximately 100mg of sucrose was weighed into 100ml glass syringes (Haberle, Labortechnik) fitted with a length of silicone rubber tubing and plastic clip. A weight of the sample to be analysed that equated to providing 78.4 mgN/l was then weighed in triplicate into the appropriate glass syringes:

\[
\text{Sample (mg)} = \left(\frac{1000}{\text{CP (g/kgDM)}}\right) \times 0.1875 \text{ mgCP/30ml} \times 78.4 \text{ mgN/L}
\]

Three blank syringes were also prepared which contained 100mg of sucrose but no sample. The plungers were lightly lubricated with Vaseline to create an air tight seal and ease movement, inserted into the syringe and the clip closed. The syringes were suspended from a Perspex lid and pre-warmed in a 39°C water bath for 1 hour before 30 ml of the inoculum was transferred using an automatic dispenser (Jencons perimatic GP) into each syringe through the rubber tubing. The plastic clip was closed, the position of the plunger read and the initial volume and time recorded. The syringe was then likely shaken and placed back into the water bath.
Gas production was recorded and the syringes manually shaken every half hour for the first 3 hours, then hourly until 6 hours and thereafter at 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 56, 64, and 72h according to Jessop and Herrero (1996).

If the gas volume exceeded 50ml, the reading was recorded, the syringe inverted, the plastic clip opened and the plunger gently returned to the 30ml mark. The new volume was recorded and the incubation allowed to continue.

7. Measurement of undegraded residual nitrogen

After 72 hours of incubation the syringes were emptied into 200ml flat medicinal bottles and mixed with 15ml of NDF solution. The bottles were autoclaved at 105°C for 1 hour to solubilise microbial protein. Using a 90mm Buchner funnel the contents of each bottle was poured under suction through Whatman 54 Hardened filter paper (20-25μm), the bottle rinsed three times with boiling distilled water and poured back through the filter paper before being rinsed with alcohol. The filter paper was dried overnight in a 60°C oven before being gently folded and cut with scissors into digestion tubes. 20ml of concentrated H₂SO₄ and 3 catalyst tablets were added to each tube, digested for 1 hour and then distilled in a Buchii Distillation Unit according to Kjedahl. After distillation, the contents of the receiving flask were titrated with 0.25M H₂SO₄ added in aliquots of 0.1ml using a pipette. The final nitrogen concentration was calculated by:

\[
\text{Undegraded Nitrogen (mgN)} = \text{Sample residue plus Blank syringe residue} \\
\text{plus filter paper (mgN)} \quad \text{filter paper (mgN)}
\]

It was assumed that the equivalent of 2.37mgN was incubated in each syringe, which meant that this analytical approach has an accuracy ± 0.071mgN or ± 3% Total CP.

8. Analysing the gas production data

The cumulative gas production was analysed using a non-linear procedure by fitting the model: \( \text{Gas} = A(1-\exp(-c-t)) + B(1-\exp(-c(t-lag))) \times \exp(5(t-l))/ (1+\exp(5(t-lag))) \) modified from Jessop and Herrero (1996). The original model incorporated a \( t > 1 \) function which was incompatible with the
SigmaPlot© (2002) curve fitting software used. Thus the model was modified and the t > 1 function replaced with a steep growth curve of $(\exp(5(t-l))/(1+\exp(5(t-lag)))$ (Green, 2002). This model was chosen because of the biological relevance of its parameters. A is the asymptotic gas production (ml) from fermentation of quickly degraded protein (QDP), ‘C_r’ is the fractional rate of gas production of A (fixed at > 0.3/h), ‘B’ is the asymptotic gas production (ml) from fermentation of slowly degraded protein (SDP), ‘C_r’ is the fractional rate of gas production of B (/h), ‘t’ is time (h) and ‘lag’ is the discrete time lag (h) before the fermentation of SDP begins.

In sequential order, the cumulative gas volumes were:

- Corrected to fermentation of the appropriate calculated weight of sucrose and sample
- Corrected for the fermentation of endogenous liquor material and microbial turnover. This was achieved by fitting the gas production profiles of the ‘blanks’ to the modified model of Jessop and Herrero (1996) and subtracting the mean cumulative gas production of the blanks from all gas profiles.
- Corrected for silage fermentation acid (SFA) gas production as described in Chapter 2.
- Modified by applying the formula:

  \[
  \text{Modified volume at } y \text{ (ml)} = (\text{Adjusted volume at } y \text{ (ml)} - (\text{Blank volume at } y \text{ (ml)} - \text{Blank volume at } x \text{ (ml)} + \text{Modified volume at } x \text{ (ml)})
  \]

  where \( x \) and \( y \) represent sequential time points:

- Based on the quantity of undegraded nitrogen, the modified volumes were altered to represent units of degraded protein by applying the following formula:

  \[
  \text{Proportion of CP at } 72h \text{ } n \text{ (%CP)} = \frac{(\text{Modified volume at } n \text{ (ml)} / \text{Modified volume at } 72h \text{ } n \text{ (ml)}) \times (2.37 \text{ mgN - undegraded nitrogen (mgN))}}{2.37 \text{ mgN}}
  \]

  where \( n \) represents time (\( n \)-th hour).
The protein degradation parameters of the grass silage were calculated by fitting the cumulative gas production (%CP) to the modified model of Jessop and Herrero (1996).

The effective protein degradability of each grass silage was calculated assuming an outflow rates (Kp) of 0.08/h (AFRC, 1993):

Quickly Degradable Protein (QDP) (%CP) = \( a \ (%CP) \times C_s \ (h^{-1}) \) / \( (C_s \ (h^{-1}) + 0.08) \)

Slowly Degradable Protein (SDP) (%CP) = \( (((1-(\text{lag (h)} \times 0.08)) \times b \ (%CP)) \times C_s \ (h^{-1}) \) / \( (C_s \ (h^{-1}) + 0.08)) \)

Effective Rumen Degradable Protein (ERDP) (%CP) = QDP (%CP) + SDP (%CP)

Digestible Undegraded Protein (DUP) (%CP) = \( ((0.9* (\text{CP (g/kgDM)} - (\text{QDP (%CP)* CP (g/kgDM)}) - (\text{SDP (%CP)* CP (g/kgDM)})) - 6.25) / \text{CP (g/kgDM)} \)

A.2 Why fix the rate of QCHO fermentation to >0.2h⁻¹

The volume of gas attributed to QCHO determines all subsequent estimates of SCHO and its associated parameters. A crucial component in estimating the contribution of QCHO to total gas production is the rate of QCHO fermentation \( (C_s) \), which when increased results in a lower estimate of QCHO and a higher estimated SCHO fraction (Table A.2.1).

The difference in the standard errors (SEE) of the predicted curves described using different \( C_s \) rates is within the accuracy of the technique (±0.25ml) and therefore not significantly different. The predicted volume of gas produced after 3 hours of incubation, before SCHO is assumed to be degraded, is also not significantly different between the different \( C_s \) rates (Table A.2.1). These results indicate that although the volume of gas attributed to QCHO and SCHO is dependent on \( C_s \), \( C_s \) does not affect the accuracy of the fitted curve. This leads to the next question: “What value of \( C_s \) or \( A \) is most appropriate?”

When measuring the carbohydrate fermentation dynamics of feeds using IVGPT the available carbohydrate is described as either quickly or slowly degradable. This enables diets to be formulated so that there is a close synchrony between carbohydrate and protein availability in the rumen, which has been shown to increase the efficiency of diet utilisation and microbial protein supply (Casper et al.1999). Using \( C_s \) rates of >0h⁻¹ and >0.1h⁻¹ the rates of degradation of the \( A \) and \( B \) fractions are not significantly
different (P>0.05) (Table A.2.2). This makes practical diet formulation very
difficult especially when trying to balance rations with high levels of quickly
degradable protein or when trying to reduce the risk of acidosis. By using a
$C_s$ rate of $>$0.2h$^{-1}$, which has been suggested to represent the maximum rate of
fermentation in the rumen (Russel et al., 1992), the rate of fermentation of the
$A$ and $B$ fractions are significantly different (P<0.05), the $A$ fraction is quick
and the $B$ fraction is slow (Table A.2.2).

The estimated $A$ fraction is also significantly lower using a $C_s$ rate at $>$0.2h$^{-1}$
compared to using $C_s$ rates of $>$0h$^{-1}$ and $>$0.1h$^{-1}$ (P<0.05). This has the
additional benefit that when the $A$ fraction is multiplied by a factor of 0.16
(Jessop, 2004) to account for the gas produced by protein fermentation, the
volume of gas removed is less and so to is the risk of removing carbohydrate
gas along with protein gas.

The $C_s$ parameter was therefore fixed to $>$0.2h$^{-1}$ to minimise the volume of gas
attributed to protein fermentation and to ensure that the two carbohydrate
fractions had significantly different rates.
Table A.2.1 Gas production parameters of dried grass silage (n=14) fitted using a specified $C_r$ range (h$^{-1}$). Each sample was incubated in duplicate. Standard deviation values are shown in brackets. Columns with different superscripts are significantly different (P<0.05)

<table>
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<tr>
<th></th>
<th>$C_r$&gt;0</th>
<th>$C_r$&gt;0.1</th>
<th>$C_r$&gt;0.2</th>
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</thead>
<tbody>
<tr>
<td>$A$ (ml)</td>
<td>34.45 (7.31)</td>
<td>27.55 (7.26)</td>
<td>18.19 (6.02)</td>
</tr>
<tr>
<td>SE</td>
<td>3.12 (1.95)</td>
<td>6.46 (4.54)</td>
<td>4.29 (1.84)</td>
</tr>
<tr>
<td>$C_r$ (h$^{-1}$)</td>
<td>0.0978 (0.0318)</td>
<td>0.1145 (0.0170)</td>
<td>0.2 (0.0)</td>
</tr>
<tr>
<td>SE</td>
<td>0.0156 (0.0131)</td>
<td>0.0348 (0.0232)</td>
<td>0.0740 (0.0372)</td>
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<td>$B$ (ml)</td>
<td>16.01 (7.97)</td>
<td>21.90 (5.21)</td>
<td>31.49 (5.21)</td>
</tr>
<tr>
<td>SE</td>
<td>3.47 (2.01)</td>
<td>6.45 (4.52)</td>
<td>4.28 (1.84)</td>
</tr>
<tr>
<td>$C_b$ (h$^{-1}$)</td>
<td>0.1291 (0.0529)</td>
<td>0.0938 (0.0435)</td>
<td>0.0877 (0.0251)</td>
</tr>
<tr>
<td>SE</td>
<td>0.0392 (0.0567)</td>
<td>0.0224 (0.0314)</td>
<td>0.0058 (0.0016)</td>
</tr>
<tr>
<td>Lag (h)</td>
<td>6.18 (1.80)</td>
<td>6.28 (1.54)</td>
<td>5.64 (1.34)</td>
</tr>
<tr>
<td>SE</td>
<td>0.79 (1.33)</td>
<td>0.40 (0.13)</td>
<td>0.32 (0.09)</td>
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<tr>
<td>SEE</td>
<td>0.54 (0.10)</td>
<td>0.58 (0.15)</td>
<td>0.67 (0.14)</td>
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<tr>
<td>Volume @ 3h</td>
<td>8.49 (2.59)</td>
<td>8.12 (2.78)</td>
<td>8.19 (2.71)</td>
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</table>

Gas production parameters obtained by fitting the gas production data to the equation: $\text{Gas} = A(1-\exp(-c_d t))+B(1-\exp(-c_b (t-\text{Lag}))) \times \exp(5(t-\text{Lag}))/(1+\exp(5(t-\text{Lag})))$ modified from Jessop and Herrero (1996). $A$ represents quickly degraded carbohydrate (QCHO) (g/kgDM), $c_d$ the fractional rate of gas production of QCHO, $B$ represents slowly degraded carbohydrate (SCHO), $c_b$ the fractional rate of gas production of SCHO (h$^{-1}$), $t$ is time (h) and Lag the lag phase before the fermentation of SCHO begins (h).

Table A.2.2 Comparison of the $C_r$ and $C_b$ parameters of dried grass silage (n=14) when fitted using a specified $C_r$ range. Each sample was incubated in duplicate. Standard deviation values are shown in brackets.

<table>
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<tr>
<th>Rate (h$^{-1}$)</th>
<th>$C_r$ (h$^{-1}$)</th>
<th>$C_b$ (h$^{-1}$)</th>
<th>Sig$^2$</th>
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<tr>
<td>$C_r$&gt;0</td>
<td>0.0978 (0.0318)</td>
<td>0.1291 (0.0529)</td>
<td>NS</td>
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<td>$C_r$&gt;0.1</td>
<td>0.1145 (0.0170)</td>
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<td>$C_r$&gt;0.2</td>
<td>0.2000 (0.0)</td>
<td>0.0877 (0.0251)</td>
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1. Gas production parameters obtained by fitting the gas production data to the equation: $\text{Gas} = A(1-\exp(-c_d t))+B(1-\exp(-c_b (t-\text{Lag}))) \times \exp(5(t-\text{Lag}))/(1+\exp(5(t-\text{Lag})))$ modified from Jessop and Herrero (1996).

2. NS: Not significant; * P<0.05; ** P<0.01; *** P<0.001
# A.3 Silage ID numbers and collection dates

Table A.3.1 ID number and collection dates of silages used in IVGPT experiments.

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