Institut für Tierwissenschaften

der Rheinischen Friedrich-Wilhelms-Universität Bonn

Optimising nitrogen use in dairy farming:

Evaluation of ruminal crude protein degradation and protein value of forages

Inaugural-Dissertation

zur

Erlangung des Grades

Doktor der Agrarwissenschaften

(Dr. agr.)

der

Hohen Landwirtschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität

zu Bonn

vorgelegt im August 2011

von

B. Sc. (hons.) Bronwyn Lee Edmunds

aus

Bridgetown, Australien

Referent: **Prof. Karl-Heinz Südekum**

Korreferent: **PD Dr. Jürgen Schellberg**

Tag der mündlichen Prüfung: **22. Dezember 2011**

Erscheinnungsjahr: **2012**

Summary

Nitrogen (N) utilisation in dairy farms is inefficient. High N inputs through expensive protein concentrates and fertilisers are not in balance with agricultural outputs (reproduction, growth, milk). Large losses of N occur through animal excretion and its poor management, as well as leaching from fertilised soils, leading to environmental pollution. Nitrogen inputs are also expensive to farmers and the cheapest sources are often imported soy products. A high dependency on imports, however, is detrimental to the environment and economy, thus local sources of protein, such as forages, are preferable. Combining these factors it appears that a major goal of dairy farming is to decrease N inputs and increase efficiency of N utilisation.

One crucial step in achieving this goal is to accurately assess and quantify the amino acid supply and requirement of dairy cows. This is much easier said than done as dietary crude protein (CP) is altered qualitatively and quantitatively by rumen microbes. The present study has focussed on assessing the CP quality of fresh and conserved forages, in particular focussing on post rumen quality and quantity of undegraded feed CP and total CP passing to the intestines, where amino acid absorption occurs.

The first part of the study attempted to improve the accuracy of estimation of the proportion of feed CP escaping degradation in the rumen (RUP), using *in situ* and *in vitro* methods. The *in situ* method is the most commonly used and accepted method. However, it is prone to error and a large source of it comes from colonisation of the residues by rumen microbes. This is particularly problematic for forage evaluation as microbial CP can compile over half of the residue CP, thus greatly overestimating RUP. In this study a novel combination of existing methods was used to correct residues for microbial attachment. The results were promising and further validation and standardisation would be highly beneficial for future analyses using the *in situ* technique. The *in vitro* methods used were: CP fractionation according to the Cornell net carbohydrate and protein system (CNCPS), and enzymatic degradation using the protease *Streptomyces griseus*. Both *in vitro* methods show high potential for future routine analysis of forage RUP and the results here support this further, though further research is required. The CP fractionation method requires a much larger data bank before robust regression equations can be formulated for RUP estimation. The *S. griseus* method estimated the RUP of most samples to a high degree of accuracy and further validation is also required.

The second part of the study applied the recently developed modified Hohenheim gas test as a new, rapid and simple method of assessing the protein value of forages. Utilisable crude protein (uCP) is the accepted measure of protein value in Germany and it is defined as the sum of microbial CP (MCP) and RUP at the duodenum. Multiplication by constants for amino acid content and intestinal digestibility convert uCP to the more internationally used value, metabolisable protein (MP), which more accurately describes the amino acid supply to the animal. The problem in estimating MP in most international systems is that it requires separate estimates of MCP and RUP and current methods in estimating these variable have large inherent sources of error. The modified Hohenheim gas test provides a direct estimate of combined MCP and RUP and, as it involves incubation in rumen fluid, it is sensitive to the degradation characteristics and interactions of individual feedstuffs. The results were very promising; further validation with a larger variety of feedstuffs and with *in vivo* data is required.

The third part of the study draws attention to how conservation methods could be employed to improve the protein value of forages. Grass silage was pre-wilted to four levels of dry matter (DM: 20, 35, 50 and 65%) at two rates (fast, slow) and the effect on CP degradability, protein value and amino acid content was observed. The protein quality was improved by fast wilting. Furthermore, wilting to 65% DM increased the level of RUP and decreased the non-protein N concentration. Utilisable CP was significantly improved by fast wilting, but not by increasing DM however; this is probably due to the decreased content of metabolisable energy. Finally, there was a large treatment effect on the amino acid profile of the silages. However, these effects were mostly lost after incubation in the rumen. Animal performance trials as well as a repetition of the experiment under practical ensiling conditions are recommended.

Overall, the results of these studies support those of many others showing that protein quality and supply from forages can be improved through methods used for conservation. The improvement of *in vitro* methods will aid in leading to higher levels of accuracy in estimating duodenal CP supply as well as presenting a range of other benefits such as reduced labour and financial expenditure and improved animal welfare through decreased requirement for experimental animals.

Zusammenfassung

Stickstoff (N) wird in Milchviehbetrieben nicht optimal genutzt. Hohe N-Einfuhren durch Eiweißfuttermittel und Düngemittel stehen nicht im Gleichgewicht mit landwirtschaftlich erzeugten Produkten (Reproduktion, Wachstum, Milch) und deren Ausfuhren aus dem Betrieb. Erhebliche N-Verluste entstehen durch tierische Ausscheidungen und ineffizientes Management, sowie durch Emissionen aus gedüngten Böden und dies trägt zur Umweltbelastung bei. Des Weiteren belasten N-Überschüsse das Betriebsergebnis negativ. Somit ist ein Ziel bei der Milchproduktion, die N-Einträge zu senken und dadurch die N- Ausnutzung zu steigern.

Zur Erreichung dieses Ziels müssen die Aminosäureversorgung und der -bedarf der Milchkühe genauer quantifiziert werden. Dies ist schwierig, da das Rohprotein (XP) aus dem Futter qualitativ und quantitativ durch Pansenmikroben verändert wird. Grünlandaufwüchse liefern regional erzeugte und kostengünstige Futtermittel mit einem großen Potenzial für eine bessere N Nutzung bei Wiederkäuern. Die vorliegende Studie konzentrierte sich auf die Beurteilung der XP-Qualität von frischen und konservierten Grünlandaufwüchsen, wofür verschiedene methodische Ansätze verwendet wurden.

Ziel des ersten Teils der Studie war es, eine verbesserte Schätzgenauigkeit des im Pansen nicht abgebauten Futter-XP (ruminally undegraded dietary crude protein, UDP) zu erreichen. Dazu wurden *in situ*- und *in vitro*-Methoden genutzt. Die *in situ*-Methode, obwohl weltweit verbreitet und anerkannt, ist anfällig für Fehler. Eine große Fehlerquelle stellt die mikrobielle Besiedelung der *in situ*-Residuen im Pansen dar, was zu einer Unterschätzung des ruminalen XP-Abbaus führt. In dieser Studie wurde eine neuartige Kombination vorhandener Methoden verwendet, mit der die mikrobielle Besiedelung gezielt korrigiert werden konnte. Die Ergebnisse waren plausibel, eine Validierung sowie Standardisierung für zukünftige Anwendungen sollten die nächsten Schritte sein. Die verwendeten *in vitro*-Methoden beinhalteten: Eine XP-Fraktionierung gemäß dem „Cornell net carbohydrate and protein system“ (CNCPS) sowie den enzymatischen Abbau mithilfe einer *Streptomyces griseus*-Protease. Beide *in vitro*-Methoden hatten ein großes Potential für zukünftige Routineanalysen. Die XP-Fraktionierung erfordert eine größere Grobfutter-Datenbasis, bevor robuste Regressionsgleichungen für die UDP-Schätzung abgeleitet werden können. Mit der *S. griseus*-Methode wurden die UDP-Gehalte in den meisten

Proben mit einer hohen Genauigkeit geschätzt, aber auch hier sind weitere Validierungen erforderlich.

Der zweite Teil der Studie verwendete den modifizierten Hohenheimer Futterwerttest (HFT) als eine neue, schnelle und einfache Methode zur Bewertung des Proteinwertes von Grünlandaufwüchsen anhand des im deutschen Proteinbewertungssystem zentralen Kriteriums „nutzbares Rohprotein am Duodenum“ (nXP). Das nXP stellt die Summe aus im Pansen synthetisiertem mikrobiellem XP (MXP) und UDP am Duodenum dar. Durch die Erweiterung der Schätzgleichung mit Konstanten für die Konzentrationen an Aminosäuren im nXP und dessen intestinale Verdaulichkeit kann der nXP-Wert zur international gebräuchlicheren Kenngröße umsetzbares Protein (metabolisable protein, MP) umgewandelt werden. Das Problem bei der Schätzung von MP ist in den meisten Systemen, dass separate Schätzungen des MXP und des UDP erforderlich sind und die aktuellen Methoden für die Schätzung dieser Variablen große inhärente Fehlerquellen haben. Der modifizierte HFT ermöglicht demgegenüber eine direkte Schätzung des nXP. Die Ergebnisse waren plausibel und sollten mit einer größeren Vielfalt an Futtermitteln und *in vivo* Daten validiert werden.

Im dritten Teil der Studie wurden unterschiedliche Methoden der Grünfutterkonservierung hinsichtlich ihrer Auswirkungen auf den Proteinwert von Grünlandaufwüchsen untersucht. Grüngut wurde vor der Silierung auf vier verschiedene Trockenmassegehalte (TM: 20, 35, 50 und 65 %) bei zwei Geschwindigkeiten (schnell, langsam) angewelkt und an den Silagen die Auswirkungen dieser Vorgehensweisen auf den XP-Abbau im Pansen, den Proteinwert und die Aminosäuregehalte beobachtet. Schnelles Anwelken verbesserte die Proteinqualität. Anwelken auf 65 % TM erhöhte das UDP-Niveau und verringerte die Nicht-Protein-N-Konzentrationen. Die nXP-Gehalte wurden durch die Erhöhung der TM-Gehalte nicht beeinflusst. Dies ist wahrscheinlich auf die verringerten Gehalte an umsetzbarer Energie zurückzuführen. Studien zur tierischen Leistung sowie eine Wiederholung des Experiments unter Praxisbedingungen werden empfohlen.

Die Ergebnisse dieser Studie belegen, dass Proteinqualität und die Proteinversorgung aus Futterpflanzen durch die Art der Konservierungsmethode verbessert werden kann. Eine verbesserte Vorhersagegenauigkeit der Proteinqualität von Grünlandaufwüchsen durch standardisierte *in vitro*-Messungen kann somit dazu beitragen, N-Bilanzüberschüsse von Milchviehbetrieben auszugleichen und die Milcherzeugung zu optimieren.

Table of contents

Figures

Chapter 3

Chapter 4

Chapter 5

Tables

Chapter 3

Chapter 4

Chapter 5

Abbreviations

200-, 350-, 500-, 650-F/S	Silage wilted to 200, 350, 500 or 650g of DM with either a fast (F) or slow (S) rate of moisture loss
a	Proportion of CP solubilised at initiation of incubation *in situ*
A	NPN – N that is not precipitable with tungstic acid
AA	Amino acid/s
AAT_N	AA absorbed in the small intestine
AD	Acid detergent
ADF	Acid detergent fibre expressed inclusive of residual ash
ADIN	Acid detergent insoluble N
ADFom	Acid detergent fibre expressed exclusive of residual ash
A_{max}	The maximum extent of microbial attachment at time = ∞
b	Proportion of CP insoluble but degradable in the rumen – *in situ*
B1	Soluble TP – soluble in buffer but precipitable in tungstic acid – rapidly degraded in the rumen
B2	Insoluble TP – insoluble in buffer but soluble in ND solution – degraded at an intermediate rate in the rumen
B3	NDIN - ND insoluble TP but soluble in AD – slowly degraded in the rumen
c	Rate of degradation of b – *in situ*
C	ADIN – insoluble in AD - indigestible
CA	Crude ash
CNCPS	Cornell net carbohydrate and protein system
CL	Crude lipids
CP	Crude protein
DM	Dry matter
DMI	Dry matter intake
DOM	Digestible OM
DVE	Intestinally digestible protein
FiM	Feed into Milk
FOM	Fermentable OM
FOMr	FOM in the rumen
GP	Gas production in 24 hours from 200 mg (DM) substrate

HGT	Hohenheim gas test
IS	*In situ* procedure
K_d	Rate of degradation in the rumen
K_p	Rate of passage of solids through the rumen
L	Lag – amount of time between initiation of incubation and degradation *in situ*
MA	Microbial attachment as a percentage of residue N
MCP	Microbial CP
MG1	Meadow grass sample 1
MG2	Meadow grass sample 2
ME	Metabolisable energy
modHGT	Modified Hohenheim gas test
MP	Metabolisable protein
N	Nitrogen
NAN	Non-ammonia N
ND	Neutral detergent
NDF	Neutral detergent fibre assayed without heat stable amylase and expressed inclusive of residual ash
NDIN	Neutral detergent insoluble N
NPN	Non-protein N
OEB	Rumen degraded protein balance
OM	Organic matter
PADF	ADF analysed using the CP fractionation procedure
PDI	Amino N absorbed in the small intestine
PNDF	NDF analysed using the CP fractionation procedure
PF	CP fractionation
R	Rate of attachment of microbes to feed particles in the rumen
RUP	Ruminally undegradable feed CP
SGP	*Streptomyces griseus* procedure
TP	True protein – that is precipitable in tungstic acid
u	Proportion of CP that is insoluble in the rumen – *in situ*
uCP	Utilisable crude protein at the duodenum

CHAPTER 1 GENERAL INTRODUCTION

Environmental pollution from agriculturally associated nitrogen (N) is the result of unbalanced N supply, demand and utilisation. A significant contributor to N pollution is dairy farming. Global ammonia (NH_3) emissions from ruminants surpass all other domestic species (Bouwman et al., 1997). Ammonia is produced and evaporated from urinary excretion and contributes to ecosystem acidification and eutrophication (Galloway and Cowling, 2002). Use of mineral fertilisers is the second largest contributor to global ammonia emissions, after domestic animals (Bouwman et al., 1997). Application of mineral N-fertiliser and manure to the soil is also largely responsible for increased nitrous oxide (N_2O) emissions, which contribute to tropospheric warming and stratospheric ozone depletion, and nitrate (NO_3) pollution in soils and water systems through leaching (Tamminga, 2003). Generally, the utilisation of N in intensive dairy farming is highly inefficient with large inputs, large amounts of waste, and only a fraction of the dietary N supply being converted into milk protein. To add further insult to injury, protein is usually the most expensive component of dairy cow diets, particularly as many supplements are imported, a factor in itself which is by no means environmentally friendly.

Ideally, dairy cow requirements should be met by their natural feed; forage. High production demands and limited space however make high energy and protein supplements an essential part of the diet. The problem is not always a factor of low crude protein (CP) from forages but rather poor efficiency of CP utilisation. For example, grass silage provides a high proportion of dietary CP but production responses are improved with added protein supplements. The inefficient utilisation of silage CP is based largely on the composition of N components (Givens et al., 2004). A high proportion of forage true protein is degraded during ensiling to form non-protein N, which is rapidly degraded to ammonia in the rumen. A significant portion of this is excreted due to insufficient rapidly available energy to the microbes to capture the ammonia-N. Rumen degradation is highly dependent on the structure of N components and this may be altered by the type of conservation (hay vs. silage) and methods used within conservation type, such as pre-ensiling wilting. Thus a more clear understanding of the effect of forage conservation on protein quality will aid in improving the efficiency of N utilisation by ruminants and decrease dependency on expensive, protein rich supplements.

In order to meet growth, reproduction and, in particular, lactation demands ruminants must receive an adequate supply of absorbable amino acids. Metabolisable protein (MP) is the most commonly used and accepted measure of duodenal protein supply and compiles

microbial CP (MCP) and undegraded feed CP (RUP), or more accurately, amino acids, that can be digested in the small intestine. Both portions are important in terms of amino acid composition. Microbial amino acids make up the majority of duodenal supply and their composition is generally quite uniform (Storm and Ørskov, 1983), though minor variations do occur with diet type (Boisen et al., 2000). Any deficit in requirement must be met by RUP. Unfortunately, both MCP and RUP are exceedingly difficult to accurately measure because the ingested dietary amino acid supply is altered both quantitatively and qualitatively in the rumen by microbial action. Whilst the N requirement of rumen microbes must be met, simply increasing the concentration of dietary CP will often lead to a higher level of N excretion and to the previously described environmental and economical problems. On the other hand, insufficient ruminally available N can suppress microbial growth (Russell et al., 1992) and diminish the supply of MP to the animal. Clearly a balance must be found that optimises efficiency, however the complicated, interacting processes occurring in the rumen and affecting microbial production make this a challenging goal.

Essentially, an ideal protein evaluation system should accurately quantify the proportion of both MCP and RUP in MP as well as the requirements by rumen microbes of rumen degradable protein (RDP) and by the host animal of MP. Improvement in protein evaluation systems have aided in more accurately quantifying these variables but a higher level of accuracy is required. Currently used methods still rely heavily on fistulated animals. Besides the obvious ethical concerns, *in situ* and *in vivo* methods are associated with high costs in money, labour and time. Furthermore they are difficult to standardise and bring about high levels of error (Nocek, 1988). *In vitro* methods are preferable; however they must first be validated against *in vivo* or *in situ* data.

References

Bouwman, A. F., Lee, D. S., Asman, W.A.H., Dentener, F. J., Van Der Hoek, K. W., Olivier, J. G. J. (1997) A global high-resolution emission inventory for ammonia. *Global Biochemical Cycles* 11, 561-587

Cowling, E. B. and Galloway, J. N. (2001) Challenges and opportunities facing animal agriculture: optimising nitrogen management in the atmosphere and biosphere of the Earth. *Journal of Animal Science* 80 (E. Suppl. 2), E157-1E67

Givens, D. I., Rulquin, H. (2004) Utilisation by ruminants of nitrogen components in silage-based diets. *Animal Feed Science and Technology* 114, 1-8

Nocek, J. E. (1988) In situ and other methods to estimate ruminal protein and energy digestibility: A review. *Journal of Dairy Science* 71, 2051-2069

Russell, J. B., O'Connor, J. D., Fox, D. G., Van Soest, P. J., Sniffen, C. J. (1992) A net carbohydrate and protein system for evaluating cattle diets: I. Ruminal fermentation. *Journal of Animal Science* 70, 3551-3561

Storm E. and Ørskov E. R. (1983) The nutritive value of rumen micro-organisms in ruminants. *British Journal of Nutrition*, 50, 463-470.

Tamminga, S. (2003) Pollution due to nutrient losses and its control in European animal production. *Livestock Production Science* 84, 101-111

CHAPTER 2 SCOPE OF THE THESIS

This is a cumulative thesis composed of three papers directly or indirectly addressing the problems mentioned in the general introduction. The third to fifth chapters compile manuscripts that are formatted according to the regulations of the journal chosen for submission.

The third chapter focuses on measuring RUP using a variety of techniques. The *in situ* method is the most widely accepted method in analysis of RUP, however it is very difficult to standardise, results are highly variable, and it requires access to surgically modified animals. It is, however, the accepted standard in estimating RUP and was thus used in this study with an additional improvement. Many attempts have been and are being made to accurately predict RUP using *in vitro* methods, as they are generally much easier to standardise and reproduce results as well as having ethical advantages. Two well researched methods were chosen and recent improvements were applied in an attempt to improve the accuracy of RUP prediction of forages and identify further steps to be taken.

The fourth chapter applies a recently developed *in vitro* method which provides a direct estimate of utilisable CP at the duodenum. Utilisable CP is simply the sum of MCP and RUP at the duodenum and is the accepted estimate of protein value in Germany. It can be converted to MP using constants for amino acid concentration and digestibility. A direct measurement reduces the number of variables required, as would be necessary for an indirect estimation, and their associated prediction error, providing a theoretically more accurate quantification. The new method was validated against the currently used method in Germany.

The fifth chapter focuses on using conservation methods to improve protein quality. More specifically: the effect of wilting grass on protein quality and amino acid composition of silage. Techniques from the previous two chapters were applied as well as a full amino acid analysis of the silages before and after incubation in the rumen.

Extensive additional analyses to those reported were conducted in the course of this PhD. The results were selected based on their quality and usefulness in furthering our understanding and assessment of protein quality in dairy farming.

CHAPTER 3

Estimating ruminal crude protein degradation of forages using *in situ* and *in vitro* techniques

B. Edmunds[1,2], K.-H. Südekum[1], H. Spiekers[2], F.J. Schwarz[3]

[1]*Institute of Animal Science, University of Bonn, Endenicher Allee 15, 53115 Bonn, Germany*

[2]*Institute for Animal Nutrition and Feed Management, Bavarian State Research Centre for Agriculture (LfL), Prof.-Dürrwaechter-Platz 3, 85586 Poing, Germany*

[3]*Animal Nutrition Weihenstephan, Technical University of Munich, Weihenstephaner Berg 3, 85350 Freising, Germany*

Submitted for publication

Abstract

Feed crude protein (CP) escaping rumen degradation (RUP) from a variety of forages was estimated using two *in vitro* procedures: the Cornell protein fractionation procedure and enzymatic degradation procedure by *Streptomyces griseus* protease. Some recent improvements to both *in vitro* procedures were applied. The *in situ* technique served as a reference method and a novel combination of methods was used to correct for microbial colonisation of residues. Twenty-five forages, varying in conservation type (fresh, ensiled, dried) were analysed. Assumed passage rates of 0.02, 0.04 and 0.06 h^{-1} were applied to estimates of RUP using *in situ* and chemical fractionation. Results from both *in vitro* procedures correlated linearly with *in situ* values ($P<0.05$). Enzymatic degradation was the more accurate method in estimating *in situ* RUP ($r^2 = 0.69$, $P<0.0001$). In both *in vitro* procedures, silage and dried forage were more accurately estimated than freshly harvested forage, which may be explained by the higher and more variable concentration of intermediately degraded CP fraction B2 in the latter. Estimation of RUP from CP fractions needs further improvement. The results imply that *in vitro* procedures may soon be applied for routine analysis of RUP in forages with a higher level of confidence.

Keywords: crude protein fractionation, *Streptomyces griseus* protease, methods, forage conservation, microbial contamination

Introduction

Crude protein (CP) degradation in the rumen is a very complex process and its quantification is an ongoing challenge to ruminant nutritionists. Ruminally undegraded feed CP (RUP) for individual feed products is required by most feed evaluation systems in the calculation of metabolisable protein (NRC, 2001; Tamminga et al., 2007; Volden, 2011). Moreover, constant attempts to increase RUP supply to the intestines of high producing dairy cows call for accurate, reproducible and simple methods for routine analysis. The determination of RUP *in vivo* is too expensive, time-consuming and labour-intensive for routine analysis and animal welfare is questionable (Stern et al., 1997).

In situ techniques mimic *in vivo* conditions and are widely adopted as the standard method for analysing RUP as well as providing reference values against which *in vitro* techniques are correlated. However, the procedure is prone to various sources of error, one of which is colonisation of the residues by rumen microbes. Microbial markers such as diaminopimelic acid (DAPA) or mechanical pummelling are often used as a means of correction, although the former technique is not accurate for reasons described by Tedeschi et al. (2001) and the latter requires access to a stomacher. The commonly used purine analysis method (Zinn and Owens, 1996) has also been shown to be inaccurate for reasons described by Klopfenstein et al. (2001). For these reasons, and in circumstances where markers have not been or can not be used, a simple and standardised method is required. Mass et al. (1999) reported that microbial matter is soluble in neutral detergent solution. Provided no feed-associated neutral detergent soluble material remains, this knowledge can be used to remove microbes from residue. However, for the calculation of effective degradability the *in situ* procedure requires some short incubation times (< 16 h) in which not all neutral detergent soluble material is degraded. Krawielitzki et al. (2006) found that microbial colonisation of residues was exponential with time and provided an equation, which required a variable describing the maximum attachment of microbes at saturation point. In the following study, maximum microbial attachment at saturation point was estimated by boiling in neutral detergent solution (Mass et al., 1999) and the results applied to the equation of Krawielitzki et al. (2006). The corrected *in situ* RUP served as reference values.

Although the *in situ* method is widely used as the reference method in estimating RUP, it is labour-intensive and requires access to rumen cannulated animals. Based on these problems, *in vitro* methods are desirable for routine analysis of protein degradability. Two methods used currently include CP fractionation according to the Cornell Net Carbohydrate and Protein

System (CNCPS: Sniffen et al., 1992) and enzymatic degradation by commercial proteases (Krishnamoorthy et al., 1983). Estimation of RUP by these methods is still unreliable and requires refinement and standardisation. The main source of error in the CP fractionation system is the relatively low reproducibility of the CP degradation rate, which is highly influential on the level of CP escaping rumen degradation. In an attempt to bypass the need to estimate CP degradation rate, Shannak et al. (2000) developed regression equations from *in situ* RUP values using CP and fibre fractions. These equations, however, are validated for concentrates and were proven inaccurate for forages (Kirchhof, 2007). New equations for forages were developed (Kirchhof, 2007) and have been used in this study. Regarding *in vitro* studies with protease, many variants of the procedure have been used, making interpretation of results very difficult. An attempted standardisation of the procedure using *Streptomyces griseus* protease (SGP) was presented by Licitra et al. (1998, 1999) and these recommendations have been applied here.

The main objective of the present study was to apply this new information to the *in situ* and *in vitro* (CP fractionation and SGP) methods and to assess their accuracy in estimating the RUP of forages. An additional objective was to gain a better understanding of the suitability of these techniques in assessing a variety of forage types (fresh, ensiled, dried).

Materials and Methods

Animals

For the *in situ* trial six non-lactating German Holstein cows (≈ 750 kg body weight) were used. Cows were fitted with a 10 cm diameter rumen cannula (#2C, Bar Diamond Inc., Parma ID, USA) and housed side by side in a 20℃ climate controlled stall. All animals were tethered to individual feeding troughs and allowed *ad libitum* access to water. Feeding occurred at 07:00 and 16:00 h daily in two equal meals meeting maintenance requirements. The ration was composed of approximately 0.22 soybean meal and mineral concentrate, 0.53 maize silage and 0.26 grass hay as a proportion of total DM. The hay was given 30 minutes after the concentrate/silage mix. The diet was started 14 days before the trial and finished on the day of the last incubation.

Feedstuffs

From southern Germany twenty-five forage samples (Table 1) from harvest year 2008 were selected to cover a range of conservation methods and species. Samples included: freshly harvested (n = 12), silage (n = 8), hay (n = 1) and artificially dried (n = 4). Of the freshly

harvested forages two of the following samples from the same parent material were taken: white clover (*Trifolium repens*), perennial ryegrass (*Lolium perenne*), ryegrass/white clover sown field and meadow grass from two fields located approximately 50 km apart (field 1: approximately 0.80 grass, 0.15 legumes, 0.05 herbs; field 2: approximately 0.80 grass, 0.10 legumes, 0.10 herbs). Two lucerne samples were also taken; however these came from different sources i.e. not the same parent material. All fresh samples were frozen within two hours of harvesting except for meadow grass-field 2 and lucerne, which were collected from the drying plant directly before drying and used to compare the effects of artificial drying on CP degradability. The material had been field wilted prior to transportation to the drying plant (see Table 1 for wilting times). Once collected these samples were also frozen.

Eight silage samples were obtained from the same parent material in a controlled trial investigating the effects of wilting extent and speed on ruminal CP degradation (results to be published in a future paper). Meadow grass (approximately 0.80 grass, 0.10 legumes and 0.10 herbs; second harvest, heading) was subdivided and wilted in the sun (fast) or shade (slow) to 200, 350, 500 and 650 g/kg DM. The grass was then ensiled in triplicate, without additives, in 1.75 l glass jars according to the scheme for silage testing (Bundesarbeitskreis Futterkonservierung, 2006). The trial was conducted at the Landwirtschaftliches Zentrum Baden-Württemberg (LAZBW), Aulendorf, Germany.

The hay and artificially dried samples have been combined to represent dried forages. The single hay sample originated from meadow grass-field 1 (first harvest) in a controlled trial. A subsample of the grass was field wilted for 56 hours with manual turning to promote even drying. Once a dry matter (DM) content of > 850 g/kg was reached, the hay was stored in a cool room at 4°C.

Of the four artificially dried samples, two were derived from meadow grass-field 2 and one from each lucerne sample. Processing took place in different factories. The meadow grass was chopped to a theoretical length of 0.5 cm, dried (entrance temperature 350°C, exit temperature 98°C) milled and pelleted. The lucerne samples were chopped to a theoretical length of 1 cm and dried (entrance temperature 540°C and 410°C and exit temperature 112°C and 109°C for harvest 1 and 3 respectively) without subsequent processing. Drying time for both forage types was about eight minutes and the starting and finishing temperature depended on the water content of the material.

General analysis

All samples were freeze-dried and milled through a 3 mm screen for the *in situ* trial and a 1 mm screen for all other analyses. Proximate analysis was done according to the German Handbook of Agricultural Experimental and Analytical Methods (VDLUFA, 2004) and method numbers are given. The DM of the forages and incubation residues was determined by oven-drying of a subsample at 105°C (3.1). Ash and crude lipids (CL) were analysed using methods 8.1 and 5.1.1 respectively. Crude protein was determined by Dumas combustion (4.1.2) for original forage material and *in situ* residues and by Kjeldahl (4.1.1) for CP fractionation analysis using Vapodest 50s carousel (Gerhardt, Königswinter, Germany) for automated distillation and titration. Neutral detergent fibre (6.5.1; assayed without heat stable amylase) and acid detergent fibre (ADF; 6.5.2) are expressed inclusive of residual ash. Additionally, ADF without residual ash (ADFom) was estimated by NIRS for use as a variable in calculating metabolisable energy (ME: GfE, 2008; see Table 1 for calculation).

In situ procedure

Rumen CP degradability was measured in an *in situ* trial conducted at the Department of Animal Nutrition of the Technical University of Munich (TUM), Freising-Weihenstephan. The procedure followed the basic method of Madsen and Hvelplund (1994) and Shannak et al. (2000) with the following alterations and specifications. In preparation, 4 ± 0.05 g of feedstuff was weighed into 10 x 20 cm Polyester-Monofilament (N-free) bags, pore size 50 ± 15 µm (R1020. Ankom Technology, Macedon, NY, USA). Bags were incubated at least in triplicate in the ventral rumen of three cows; the number or replications depending on incubation time, expected degradability, and amount of residual material required for post-incubation analyses. Plastic cable ties were used to seal the bags and attach them to an 800 g cylindrical plastic weight used for incubation. The weight was attached to the inside of the fistula with an 80 cm long line. All incubations began at 06:30 h, 30 minutes before the morning feed. Incubation periods were 2, 4, 8, 16, 24, 48 and 96 h. Directly after removal, all bags were immersed in ice-water to inhibit further microbial action and then washed by hand and then in a washing machine using cold water (without soap or spinning) with two water changes for 20 minutes. The bags were then freeze-dried, allowed to equilibrate to air moisture, and reweighed. Incubation residues were pooled per cow and incubation time. Three bags per feedstuff were also allocated for the calculation of the 0 h washout fraction. Disappearances were determined by following the same machine washing procedure as for the incubated bags. Water soluble material was estimated by mixing duplicate samples in 100

ml, 40°C distilled water and then filtering through No. $595^{1/2}$, diameter 270 mm filter paper (Schleicher and Schuell, Dassel, Germany). Water-insoluble CP escaping as small particles (SP) was estimated by subtracting water-soluble (WS) CP from 0 h CP washout. The equation of Hvelplund and Weisbjerg (2000) was used to correct (C) the CP disappearance (DI) for small particle loss for each feedstuff (i) at each incubation time point:

$$CDI_i = DI_i - SP\ (1 - ((DI_i - (SP + WS)) / (1 - (SP + WS)))).$$

Correction for microbial attachment (MA: mg/g residue CP) to undegraded particles was carried out using the exponential equation of Krawielitzki et al. (2006):

$$MA = A_{max}\ (1 - e^{-Rt}),$$

where A_{max} is the maximum extent of bacterial colonisation at $t \approx \infty$, R is the rate of colonisation (h^{-1}) and t denotes the incubation time (hours). The rate of microbial attachment was calculated as (Krawielitzki et al. 2006):

$$R\ (h^{-1}) = (133 + 0.09\ NDF\ (g/kg\ DM) - 0.35\ CP\ (g/kg\ DM))/1000.$$

The A_{max} was estimated by treating a subsample of the residue ($t \geq 16$ h) with neutral detergent solution with the assumption that the residues only contained cell wall bound CP (estimated from neutral detergent insoluble N; NDIN) and microbial matter was soluble in neutral detergent (Mass et al., 1999). Due to small sample size and large number of samples, fibre bags (38 µm pore size; Gerhardt, Königswinter, Germany) were used instead of manual filtration on filter paper. Duplicates of 0.5 g were boiled for 1 h in neutral detergent solution, rinsed thoroughly with distilled water, oven-dried overnight at 60°C, reweighed and analysed for CP. The difference in CP between pre- and post neutral detergent-treated residues was taken as microbial CP. The mean from the 16, 24, 48 and 96 h residues was used to represent A_{max}. Krawielitzki et al. (2006) found a significant relationship between NDF and CP content of the original material and A_{max} ($r^2 = 0.57$: $P=0.006$) where:

$$A_{max}\ (mg/g\ residue\ CP) = (506 + 0.48\ NDF\ (g/kg\ DM) - 0.77\ CP\ (g/kg\ DM)) / 10$$

Results of this calculation were compared with those of extraction by neutral detergent. The time and sample specific MA estimation was subtracted from the original residue CP before further degradability calculations were carried out.

Table 1 Feedstuff description and proximate parameters

Feedstuff	Harvest	Maturity	Wilting time	DM	CP	Ash	CL	NDF	ADF	ME[b]
			h	g/kg	----------g/kg DM----------					MJ/kg DM
Fresh										
Perennial ryegrass	1	Early head		239	109	64	21	427	225	11.0
Perennial ryegrass	3	Mid head		184	148	100	41	419	278	11.0
White clover	1	Early bud		135	241	113	15	227	235	10.9
White clover	3	Mid bud		166	276	102	21	291	238	11.3
Ryegrass/white clover	1	Early bud		237	163	89	25	391	256	11.0
Ryegrass/white clover	1	Late bud		163	148	83	21	412	-	10.7
Meadow grass 1	1	Late head		212	140	86	23	437	281	10.2
Meadow grass 1	2	Early flower		180	149	90	26	476	310	9.9
Meadow grass 2	1	Early head	5	256	184	87	29	446	212	11.4
Meadow grass 2	1	Early head	24	309	193	88	27	413	209	11.4
Lucerne	1	Mid bud	15	262	185	96	21	346	259	9.7
Lucerne	3	Early flower	12	362	191	100	19	396	330	9.3
Silage[a]										
Fast 200	2	Mid head	3	194	188	113	54	386	244	11.0
Slow 200	2	Mid head	5	193	189	114	60	376	227	11.2
Fast 350	2	Mid head	7	381	189	113	59	377	247	11.2
Slow 350	2	Mid head	31	373	191	116	54	394	254	10.8
Fast 500	2	Mid head	9	499	186	112	50	391	256	10.8
Slow 500	2	Mid head	33	466	195	117	51	386	249	10.8
Fast 650	2	Mid head	26	692	179	111	40	442	275	10.1
Slow 650	2	Mid head	50	669	191	115	41	431	275	10.0
Dried										
Meadow grass 1-hay	1	Late head	56	>830	128	70	23	513	270	10.3
Meadow grass 2	1	Early head	5	>900	190	88	40	397	205	11.5
Meadow grass 2	1	Early head	24	>900	194	87	40	380	198	11.5
Lucerne	1	Mid bud	15	>900	206	111	25	395	304	9.4
Lucerne	3	Early flower	12	>900	178	101	20	388	298	9.2

[a] Fast and slow refer to the rate the grass was wilting at and the following number refers to DM content.
[b] ME (MJ/kg DM; GfE, 2008) = 7.81 + 0.07559 GP - 0.00384 Ash + 0.00565 CP + 0.01898 CL - 0.00831 ADFom. All DM fractions are expressed as g/kg DM.
DM, CP, CL, NDF, ADF, ADFom, GP and ME are respectively dry matter, crude protein, crude fat, neutral detergent fibre (inclusive of residual ash), acid detergent fibre (inclusive of residual ash), acid detergent fibre (exclusive of residual ash), gas production at 24 hours (ml/200 mg DM) and metabolisable energy.

Time specific degradation of CP and effective CP degradability was calculated using the equation of McDonald (1981) with the modification of Wulf and Südekum (2005), which assumes that no degradation occurs during the lag phase:

$$\text{Effective degradability (g/kg CP)} = a + (bc / (c + K_p))e^{-K_pL}$$

where K_p is the rate of passage through the rumen, a is the soluble fraction, b is the insoluble but potentially rumen degradable fraction (calculated as d - a, where d is the potentially ruminal degradable fraction), c is the rate constant of disappearance of fraction b and L is the lag phase. Effective degradability was estimated at assumed passage rates (K_p) of 0.02, 0.04 and 0.06 h^{-1} (K_p2, 4, 6) to represent low, medium and high feeding amounts and typical rates of ruminal solid outflow for forages. The RUP from the *in situ* analysis was calculated as 1000-effective degradability.

Crude protein fractionation

Division of CP into five fractions (A, B1, B2, B3 and C) based on characteristics of degradability was done according to the CNCPS (Sniffen et al., 1992) using standardisations and recommendations of Licitra et al. (1996). Briefly, the A fraction is non-protein nitrogen (NPN), the B fraction is degradable true protein and the C fraction is undegradable true protein. The B fraction is further divided into B1, B2 and B3. Fraction B1 is buffer soluble and rapidly degraded in the rumen, B2 degrades at an intermediate rate and B3, which represents NDIN, degrades slowly in the rumen. All fractions, as well as CP, were analysed in triplicate and the N content was analysed using the Kjeldahl procedure. Regression equations of Kirchhof (2007) for K_p 0.02, 0.05 and 0.08 h^{-1} were used to estimate RUP from CP, CP fractions, PNDF and PADF. Subsequent linear regression of RUP values allowed prediction of K_p4 and 6. The equations are as follows:

$$\text{RUP 2} = 204.3207 + (1.0753 \times C) + (-0.0014 \times (CP \times (A + B1)))$$

$$\text{RUP 5} = 321.9023 + (0.1676 \times PADF) + (-0.0022 \times (CP \times (A + B1))) + (0.0001 \times (CP \times C^2))$$

$$\text{RUP 8} = 285.5459 + (1.2143 \times C) + (0.0005 \times (PNDF \times B2)) + (-110.1740 \times ((A + B1)/PNDF))$$

where PNDF and PADF refer to NDF and ADF estimated from the residue after boiling in the respective solution according to Licitra et al. (1996). Initially, the equations of Shannak et al. (2000) were used to calculate RUP from CP fractions. However, these equations were

validated for concentrates and are unsuitable for forages, as found by Kirchhof (2007), Kirchhof et al. (2010) and again in this study.

Enzymatic in vitro procedure

Simulated rumen protein degradation was estimated using *S. griseus* protease (5.8 U/mg) following the standardised protocol of Licitra et al. (1998). The addition of the protease solution was based on the true protein concentration of the sample and the incubation was at pH 6.7. The true protein content had already been estimated from the CP fractionation procedure (CP - NPN). An incubation period of 24 h with an enzyme activity of 0.58 U/ml, making a ratio of 24 U/g true protein, was chosen for forages based on the findings of Licitra et al. (1999). Previous studies have indicated that degradation using proportionally higher enzyme concentrations and shorter incubation times provides similar results (Coblentz et al., 1999; Licitra et al., 1999) however; the longer time was chosen to offset any lag effects and reduce the standard error between runs.

For the analysis approximately 2.5 g dried sample was accurately weighed into a conical flask and 200 ml borate-phosphate buffer (pH 6.7-6.8) was added. The flasks were then incubated for 1 h at 39°C in a shaking water bath. After 1 h the calculated amount of protease solution was added to each flask and incubation continued for another 24 h. At the end of the incubation time the flasks were removed and the whole contents filtered through fibre filter bags (38 µm pore size) using a mild vacuum and rinsed with 1.25 l distilled water. The bags were freeze-dried, weighed and analysed for CP and DM. The RUP was calculated as:

RUP (g/kg CP) = ((residual CP / initial CP in flask) x 1000) / CP

Samples were repeated in three flasks over two runs, making a total of six repeats per sample.

Statistics

The non-linear *in situ* variables *a, b, c* and *L* were estimated by SAS (SAS version 9.1) using the NLIN-procedure set to the algorithms of Marquardt (1963). Linear regression was used to compare RUP results from different methods, using the reference values (*in situ*) as the dependent variable. Relationships are described using the coefficient of determination (r^2), and root mean square error (RMSE), which describes the standard deviation of the error, and regression coefficients (slope, intercept). Proc GLM was used to analyse differences between means for non-continuous data using least squares means.

Results

Proximate analysis

Results of the proximate analysis are presented in Table 1. Dry matter, CP, ash, CL, NDF, ADF and ME were all within the expected range for the respective feedstuff.

In situ

Individual feedstuff data for microbial attachment correction variables A_{max} and rate of attachment (R) can be viewed in Table 2, where two sets of A_{max} values are presented. The measured values were generally lower than the calculated ones. Initial regression analysis revealed a significant ($P<0.05$) but weak correlation between calculated and measured values ($r^2 = 0.26$). The outlying measurements were from both white clover and third-harvest lucerne samples. Removal of these four values improved the r^2 to 0.59.

Means and standard deviations of soluble (*a*), insoluble but potentially degradable (*b*) and undegradable (*u*) fractions for the three forage types (fresh, ensiled, dried) are shown in Table 3. The *a* fraction of silage was higher than fresh and dried forage, whilst the *b* fraction was lower ($P<0.05$). There were no differences between fresh and dried forages ($P>0.05$) for *a* and *b* fractions and all forage types had the same level of undergradable CP (fraction *c*). Mean RUP values based on forage type are presented at three assumed rates of passage in Table 4. Values (K_p4 only) are presented on a per feedstuff basis in a companion paper (Edmunds et al., 2012; Chapter 4). Tabulated RUP values (Universität Hohenheim – Dokumentationsstelle, 1997) corresponding to forage type, harvest number and maturity, are also presented as a mean. The results for fresh forage, hay and silage were within the range of the tabulated data however, not at one consistent rate of passage. Most often K_p2 values resembled tabulated data but in some cases K_p4 or K_p6 was a closer match. Tabulated data for artificially dried forage estimates RUP to be approximately 400 g/kg of CP, which is 3 to 4 times more than similar fresh material. The *in situ* RUP estimates for the four artificially dried samples were only about 30% higher than their fresh counterparts.

Crude protein fractionation

Unfortunately, after the proximate and *in situ* analysis there was insufficient material from the 200 g/kg DM slow wilted silage for the *in vitro* analyses. Crude protein fractions for the three forage groups are shown in Table 3. Silage had higher A and lower B2 levels. Fraction B1 was higher in fresh forage and B2 tended to be higher in fresh than in dried forage

(P=0.089). Fractions B3 and C were higher in dried forage. Table 4 provides mean RUP values for forage groups. As a general trend, RUP from dried forage was highest followed by fresh and ensiled forage, which is the same trend as *in situ* determined RUP. Means from the entire data set were not different from *in situ* means for the corresponding passage rate (K_p2 130 vs 139, P=0.997; K_p4 184 vs 201, P=0.946; K_p6 229 vs 264, P=0.340).

Table 5 shows regression equations and statistics for each passage rate. Relationships at all assumed rates of passage were linear and significant (P<0.0001). The r^2 at all rates of passage was approximately 0.5. The slope was closest to one at K_p6, however this was still quite low (0.61) and all intercepts were different from zero (P<0.001). The RMSE was lowest at K_p2 (19.03). Study of the data within forage type at K_p4 (Figure 1) revealed that there was no correlation in RUP between CP fractionation and *in situ* methods for fresh forages (r^2 = 0.12, P>0.05) and this was due to four samples, which were all legumes. Removal of these samples improved the r^2 to 0.76 (n = 7, P=0.001) and the slope (0.94) and intercept (-24.6) approached one and zero, respectively. Using this grass-specific regression line, the two white clovers were under-predicted, even though the first-harvest sample was only 20 g/kg CP less than the *in situ* K_p4 value. Both of the lucerne samples were over-predicted by CP fractionation. Their artificially dried counterparts were also over-predicted by about 60 g/kg CP. Silages (n = 7) were slightly underestimated by CP fractionation at all passage rates; however the prediction equation was strong (r^2 = 0.91 at K_p4, P<0.0001; Figure 1). Artificially dried grass samples (n = 2) were over-predicted by approximately 30 g/kg CP at K_p4. The single hay sample was well estimated with *in situ* and CP fractionation values of 269 and 254 g/kg CP, respectively, at K_p4.

Table 2 Microbial colonisation data providing rate (R) and extent (A_{max}) of attachment as calculated by Krawielitzki et al. (2006) and mean and standard deviations of A_{max} of four *in situ* residues (t = 16, 24, 48 and 96 h) measured from treatment with neutral detergent solution.

Feedstuff	Rate (R) h^{-1}	A_{max} mg microbial CP / g residue CP		±
		Calculated	Measured	
Fresh				
Perennial ryegrass	0.13	627	564	85
Perennial ryegrass	0.12	593	521	69
White clover	0.07	429	496	55
White clover	0.06	434	365	51
Ryegrass/white clover	0.11	568	462	31
Ryegrass/white clover	0.12	590	543	108
Meadow grass 1	0.12	608	526	56
Meadow grass 1	0.12	620	535	84
Meadow grass 2	0.11	578	511	49
Meadow grass 2	0.10	556	519	86
Lucerne	0.10	530	451	104
Lucerne	0.10	549	361	55
Silage				
Fast 200	0.10	546	488	90
Slow 200	0.10	541	499	57
Fast 350	0.10	542	452	17
Slow 350	0.10	548	514	81
Fast 500	0.10	550	437	42
Slow 500	0.10	541	478	106
Fast 650	0.11	581	462	61
Slow 650	0.10	566	485	37
Dried				
Meadow grass 1- hay	0.13	654	569	83
Meadow grass 2	0.10	550	421	55
Meadow grass 2	0.10	539	436	24
Lucerne	0.10	537	382	16
Lucerne	0.11	555	282	84
mean	0.11	557	470	
st dev	0.02	49	69	

[a] Fast and slow refer to the rate the grass was wilting at and the following number refers to DM content.

Table 3 Division of crude protein fractions based on degradability *in situ* (IS) or solubility in various media (PF). Means and standard deviations for freshly harvested (n = 12), ensiled (IS: n = 8, PF: n = 7) and dried (n = 5) forages are provided.

		----------*In situ*----------			----------CP fractionation----------				
		a	b	u	A	B1	B2	B3	C
		g/kg CP							
Fresh	mean	308[a]	635[a]	57	205[a]	134[b]	523[a]	115[a]	25[a]
	±	*81*	*82*	*15*	*35*	*36*	*75*	*71*	*9*
Silage	mean	537[b]	406[b]	57	540[b]	39[a]	277[b]	118[a]	26[a]
	±	*138*	*139*	*05*	*126*	*12*	*63*	*68*	*3*
Dried	mean	244[a]	692[a]	64	199[a]	40[a]	446[a]	271[b]	42[b]
	±	*82*	*96*	*35*	*27*	*34*	*22*	*43*	*16*

a = immediately degradable (soluble) CP
b = insoluble but potentially degradable CP
u = undegradable CP

A = non protein N
B1 = rapidly degraded true protein
B2 = intermediately degraded true protein
B3 = slowly degraded true protein
C = undegradable true protein

Enzymatic degradation

Mean RUP values within forage type, generated from incubating the samples for 24 h using 0.58 U/ml protease (24 U/g true protein), are presented in Table 4. The mean RUP value (n = 24, 233 ± 75) was closest to *in situ* K_p6 (232 ± 54, excluding 200 g/kg DM slow wilted silage; P=1.0). Linear regression against all three *in situ* values yielded a significant correlation (P<0.0001; Table 5). Slopes were similar to those from the CP fractionation regression and diverged from unity with decreasing passage rate. As with CP fractionation RMSE was lowest at K_p2 (14.94). The determination coefficient was reasonable for all three comparisons with 0.69, 0.71 and 0.69 for K_p2, 4 and 6 respectively. Three samples were responsible for lowering the r^2 values: ryegrass first-harvest and the two white clover samples. Removal of these samples increased the r^2 to 0.88 at K_p4 and 6 and 0.81 in K_p2. Slopes and intercepts were also improved e.g. at K_p4 the slope increased from 0.46 to 0.58 and the intercept decreased from 77.7 to 47.2.

Further study within conservation type at K_p4, revealed again that silage and dried forage (both hay and artificially dried) were more accurately predicted than freshly harvested forage (Figure 2). Removal of the three aforementioned outlying samples, which all belonged to the

fresh category, improved the r^2 of the fresh forage (0.27 to 0.73) and greatly improved the slope (0.26 to 0.71) and intercept (120.9 to 10.6). Conserved forages (silage and dried) analysed together had an r^2 of 0.96 a slope of 0.56 and an intercept of 58.6.

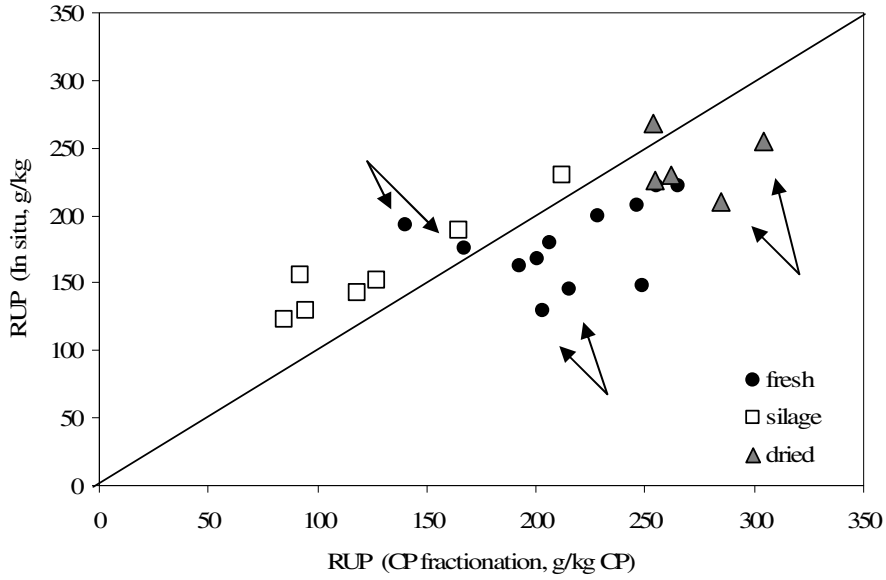


Figure 1 Linear regression of RUP (g/kg CP) estimated by crude protein fractionation (X-axis) and by *in situ* analysis (Y-axis) for freshly harvested, ensiled and dried forages at K_p4. Points highlighted with an arrow are legumes.

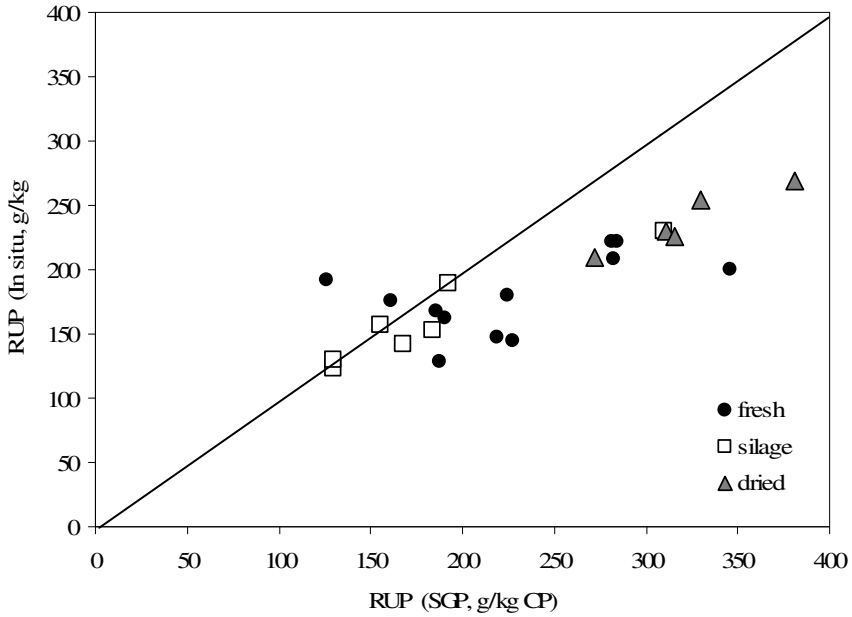


Figure 2 Linear regression of RUP (g/kg CP) estimated by enzymatic degradation (SGP; X-axis) and by *in situ* analysis (Y-axis) for freshly harvested, ensiled and dried forages at K_p4.

Table 4 Published RUP (g/kg CP) values for 24 forages (Table: Universität Hohenheim – Dokumentationsstelle, 1997) and RUP estimated by *in situ* and crude protein fractionation methods at three assumed rates of passage (K_p2, 4 and 6) and enzymatic degradation (SGP).

		Table	*In situ*			CP fractionation			SGP
			K_p2	K_p4	K_p6	K_p2	K_p4	K_p6	
Fresh	mean	154	124	179	225	151	214	276	226
	±	*33*	*20*	*30*	*39*	*27*	*37*	*48*	*62*
Silage	mean	150	116	160	196	81	128	181	181
	±	*0*	*21*	*38*	*51*	*37*	*46*	*52*	*62*
Dried[a]	mean	360	162	238	299	193	272	350	322
	±	*89*[b]	*23*	*24*	*28*	*13*	*22*	*33*	*39*

[a] Includes one hay and four artificially dried forages.

[b] Tabulated RUP values for grass hay and artificially dried forages are 200 and 400 g/kg CP, respectively (Universität Hohenheim – Dokumentationsstelle, 1997).

Table 5 Linear regression parameters of RUP from 24 forages estimated from crude protein fractionation (CPF) calculated to three rates of passage, and single time point enzymatic degradation by *S. griseus* (SGP) regressed against *in situ* estimated RUP (dependent variable) calculated to K_p2, 4 and 6.

	Slope	SE_{slope}	Intercept	$SE_{intercept}$	$P_{intercept}$	r^2	RMSE	P
CPF-2	0.38	0.08	76.38	11.79	<0.0001	0.51	19.03	< 0.0001
CPF-4	0.47	0.09	91.29	19.85	<0.0001	0.53	29.01	< 0.0001
CPF-6	0.61	0.12	109.06	25.95	0.0004	0.50	37.94	< 0.0001
SGP-2	0.29	0.04	60.89	10.12	<0.0001	0.69	14.94	< 0.0001
SGP-4	0.46	0.06	77.74	15.43	<0.0001	0.71	22.79	< 0.0001
SGP-6	0.60	0.09	93.37	20.90	0.0002	0.69	30.87	< 0.0001

Discussion

Estimation of RUP in vitro

The main objective of this study was to apply recent improvements to the CP fractionation and SGP procedures and validate them against RUP determined by the *in situ* procedure. The RUP from both *in vitro* procedures yielded strong linear relationships with *in situ* values. In terms of variation around the regression line, the SGP procedure was more accurate than CP fractionation. The results from a similar study (Gosselink et al., 2004) using 11 forages varying in conservation type failed to show a correlation between RUP analysed *in situ* and using SGP, and only a weak relationship was found using CP fractionation. This may be explained by the variations to the *in vitro* methods used. Gosselink et al. (2004) used a set amount of SGP for all forage samples, rather than sample specific amount based on true protein. Additionally, the Cornell software program was used to analyse RUP from CP fractions, rather than the regression equations used in this study.

Regarding CP fractionation, the equations of Shannak et al. (2000) were originally used to calculate RUP from CP fractions. These equations were validated mainly for concentrates (n = 29) and regression against *in situ* data in the present study yielded no or only a very weak relationship at all rates of passage. The equations of Kirchhof (2007), which were derived from grass-based forages, provided a better estimate of *in situ* data, but there was still a high amount of variation around the regression line (Table 5). The equations of Kirchhof (2007) were derived from 61 forage samples comprising 47 fresh forages from alpine pasture (0.80 grass content) of various harvests and maturities as well as seven grass hay and seven maize silage samples. These equations are thus specific for that data set and probably not very robust, particularly for the analysis of legumes. The purpose of their work was mainly to highlight that the equations of Shannak et al. (2000) were not suitable for forages. Thus, the forage-specific equations of Kirchhof (2007) were not published with the intention of widespread use for RUP estimation. Establishment of empirical equations must be based on large data sets covering a wide variety of nutritional compositions. As expected, multiple stepwise regression of the present data set resulted in equations (equations not shown) differing from those of both Shannak et al. (2000) and Kirchhof (2007) and thus reinforcing the need for a much larger data set and diversity of feedstuffs to be analysed before such equations can be developed and applied for routine forage analysis.

Regarding rumen simulated protein degradation using SGP; the results are promising for the possible use of the method in routine analysis of RUP in forages. When compared with *in*

situ values (K_p6), which correlated strongest with SGP data, ten out of the 24 samples differed by less than 20 g/kg CP from *in situ* values and five of these were under 10 g/kg. The three samples that showed the greatest deviation were at the lower and upper extremes of CP content in the sample set, which may indicate a methodological limitation. All three were fresh forages. However, in another study involving 20 forages with CP values ranging from 33 to 220 g/kg DM (Coblentz et al., 1999) this result was not observed. A slightly different technique was used by Coblentz et al. (1999) in which the pH of the buffer solution was 8.0 (rather than 6.7) and a constant ratio of enzyme activity to sample CP was maintained (rather than enzyme activity to true protein). This procedure was also used by Mathis et al. (2001) and correlation between *in vitro* and *in situ* values was strong in both studies. Because almost all previous studies using commercial protease degradation differ in their methodology, making comparisons between published data difficult, this study used methods recommended by Licitra et al. (1998, 1999). The results agreed well with *in situ* data but a comparison between the method described in this paper, and that used by Coblentz et al. (1999) would be valuable. In general, more studies using a single, standardised protocol and a large variety of forages must be generated before this procedure can be used confidently. The objective of use of RUP values in protein evaluation systems should also be considered. If a reasonably accurate ranking of the extent of degradation of feeds at high rates of passage is all that is required, then the results of this study show high potential for use of this method in routine forage analysis in the near future, particularly for conserved forages. If values representing slower rates of passage are required it may be possible to use multiple incubation times to calculate effective RUP. Degradation kinetics using commercial proteases have been attempted (Krishnamoorthy et al., 1983) however; accurately assessing the rate of degradation still requires development.

It must not be forgotten that the reference values generated from *in situ* analysis are not so reproducible (Michalet-Doreau and Ould-Bah, 1992; Schwab et al., 2005). A collaborative study by Mathis et al. (2001) with five laboratories revealed significant location based fluctuations in RUP. If this can occur in a controlled study with some standardisation, it seems likely that even higher differences could occur between institutes using variations of the *in situ* procedure. This implies that a higher level of agreement between the methods can not really be expected. The attractiveness of *in vitro* methods is that they are completely independent of the animal and thus easier to standardise. However, as validation of these methods is usually attempted against *in situ* data, a suitable standardisation of the procedure

will be difficult. Validation against *in vivo* data is also not without its problems, not only relating to cost and time but to the accuracy of the method itself.

Forage type

The second objective of this paper was to gain a better understanding of the suitability of the *in vitro* techniques in assessing various forage types (fresh, ensiled and dried). The general observation was that conserved forage is much more reliably predicted than fresh forage. In fact, based on the results of the CP fractionation procedure, a universal equation for all forages may prove difficult, if possible at all. Instead, it will probably be easier and more effective to categorise forages (e.g. fresh, ensiled and dried) with possible further subdivisions of fresh into grass and legumes and possibly in some cases even species, particularly for legumes.

Species have been shown to have a greater effect on CP composition (CP fractions) than maturity (Elizalde et al., 1999) and the present study revealed large differences between legume species. The *in situ* parameters *a, b* and *u* of the two lucerne samples analysed in this study were in good agreement with those in other studies (Elizalde et al., 1999; Julier et al., 2003) whilst results of the CP fractions were both complimentary (Eilizalde et al., 1999; Grabber, 2009) and contradictory (Yu et al., 2003; Kirchhof et al., 2010). The white clover analysed by Kirchhof et al. (2010) had much lower B1 and higher B2 content than the samples in the present study, though both studies suggest very low levels of B3, which is characteristic of white clover due to its low level of NDF. The RUP of both the white clovers was underestimated by both *in vitro* procedures. Coblentz et al. (1999) reported an overestimation in RUP of high quality legumes using an enzymatic procedure, though white clover was not included in that study. The high level of standardisation of the CP fractionation procedure suggests that contradictions in results within species are due to biochemical factors. As concluded by Kirchhof et al. (2010), a more detailed analysis of legume CP and associated cell wall composition is necessary for more accurate quantification of rumen protein degradation. Further studies are warranted using forages of highly variable composition, especially CP.

The more accurate estimation of RUP from conserved forages may be explained by the effect of conservation on CP fractions. Grabber and Coblentz (2009) showed that conservation type (silage or hay) had a larger effect on CP fractions A, B1 and B2 than polyphenols or mechanical conditioning. Fraction B2, with its intermediate rate of degradation, can have a big effect on the level of CP escaping degradation in the rumen,

particularly at faster rates of passage. Generally, when forages are conserved through ensiling or drying, there is a shift in the proportion of B1 and B2 towards A (NPN) in silage and B3 in dried products (Table 3). As NPN is already degraded and B3 is cell wall associated protein which can only be released slowly by microbial enzyme action, a better prediction of RUP from conserved forages based on either the A or B3 fractions is possible. Regression of B3 against RUP results from the present study supports this statement with 60% of the variation in RUP explained by the B3 fraction in silage and dried forages combined and zero in fresh forages (data not shown). Fresh forage has a much higher variation in B1 and B2 concentration, both between and within species, and the rate of degradation of these fractions by rumen microorganisms depends not only on their solubility and susceptibility to hydrolysis, but also on their protein structure (e.g. cross-linking and disulphide linkages; Mahadevan et al., 1980). Diversity of proteins between plant species, as well as the numerous natural and production factors (e.g. maturity, fertilisation, climate) affecting their structure and thus degradation, can make accurate estimation of rumen degradability of fresh forages, with their large B2 fraction, difficult.

Correction for microbial attachment

Regarding the method of correction for microbial colonisation of *in situ* residues; the A_{max} parameter, which is the maximum value of bacterial contamination at saturation state, was originally estimated by Krawielitzki et al. (2006) using ^{15}N as a marker. Results from that study showed maximum contamination of perennial ryegrass at 455, 622 and 849 mg/g residue CP for immature, mid-maturity and mature grass respectively. The A_{max} measured in this study was estimated by boiling in neutral detergent solution to remove microbial matter and yielded results for perennial ryegrass of 560 and 520 mg/g residue CP for mid-maturity first and second harvest respectively. An earlier, unrelated study by González et al. (1998) also described attachment of microbes to incubated feed particles using ^{15}N as being exponential and the same model as that of Krawielitzki et al. (2006) was defined. Considering the ease of obtaining estimates of $A_{max,}$ the use of neutral detergent solution appears to be an effective and simple method of correcting forage *in situ* residues for microbial contamination. This has been confirmed by Klopfenstein et al. (2001). Nevertheless, some further validation focusing particularly on legumes would be advisable as measured estimates differed somewhat from calculated estimates for most of the legume samples used in this study. It may be that legumes simply do not conform to the A_{max} equation of Krawielitzki et al. (2006).

Conclusions

In situ analysed RUP was predicted with a high level of accuracy by an enzymatic procedure using *S. griseus* protease. It is thought the good agreement could be due to a higher level of accuracy of *in situ* data due to the methods of microbial attachment used in this study as well as application of recently standardised procedures in the *S. griseus* protease method. Calculation of RUP using equations based on CP and fibre fractions has potential but requires a much larger data set to improve accuracy and robustness. Establishment of such a data base is currently underway in Germany. Conserved forages were more accurately predicted than fresh forages in both *in vitro* procedures.

Acknowledgments

This study was financially supported by the Bavarian Ministry for Agriculture and the Institute of Animal Science, University of Bonn. Thanks go out to Dr. F. Zeller and Dr. A. Metwally from TUM for their assistance with the *in situ* analysis; P. Rauch for assistance in sample collection, Dr. M. Schuster and LfL laboratory staff for proximate analysis of the samples, Dr. K. Rutzmoser for his assistance with SAS, Dr. H. Nußbaum for provision of the silages, Dr. A. Can for assistance with the enzymatic procedure.

References

Bundesarbeitskreis Futterkonservierung (Ed.) (2006): Praxishandbuch Futterkonservierung. Silagebereitung, Siliermittel, Dosiergeräte, Silofolien. 7th ed. DLG-Verlag, Frankfurt/Main, Germany.

Coblentz, W.K., Abdelgadir, I.E.O., Cochran, R.C., Fritz, J.O., Fick, W.H., Olson, K.C., Turner, J.E., 1999. Degradability of forage protein by *in situ* and *in vitro* enzymatic methods. J. Dairy Sci. 82, 343-354.

Edmunds, B., Südekum, K.-H., Spiekers, H., Schuster, M., Schwarz, F., 2012. Estimating utilisable crude protein at the duodenum, a precursor to metabolisable protein for ruminants, from forages using a modified gas test. Submitted for publication (ANIFEE-11-3704).

Elizalde, J.C., Merchen, N.R., Faulkner, D.B., 1999. *In situ* dry matter and crude protein degradation of fresh forages during the spring growth. J. Dairy Sci.82, 1978-1990.

GfE, 2008. New equations for predicting metabolisable energy of grass and maize products for ruminants. Proc. Soc. Nutr. Physiol. 17, 191-197.

González, J., Rodríguez, C.A., Andrés, S.G., Alvir, M.R., 1998. Rumen degradability and microbial contamination of fish meal and meat meal measure by the *in situ* technique. Anim. Feed Sci. Technol. 73, 71-84.

Gosselink, J.M.J., Dulphy, J.P., Poncet, C., Aufrere, J., Tamminga, S., Cone, J.W., 2004. Rumen escape nitrogen from forages in sheep: comparison of *in situ* and *in vitro* techniques using *in vivo* data. Anim. Feed Sci. Technol. 116, 35-51.

Grabber, J.H., 2009. Protein fractions in forage legumes containing protein-binding polyphenols: Freeze-drying vs. conservation as hay or silage. Anim. Feed Sci. Technol. 151, 324-329.

Grabber, J.H., Coblentz, W.K., 2009. Polyphenol, conditioning, and conservation effects on protein fractions and degradability in forage legumes. Crop Sci. 49, 1511-1522.

Hvelplund, T., Weisbjerg, M., 2000. *In situ* techniques for the estimation of protein degradability, In: Givens, D., Owen, E. (Eds.), Forage Evaluation in Ruminant Nutrition, CAB International, Walingford, UK, pp. 233-258.

Julier, B., Guines, F., Emile, J.-C., Huyghe, C., 2003. Variation in protein degradability in dried forage legumes. Anim. Res. 52, 401-412.

Kirchhof, S., 2007. Kinetik des ruminalen *in situ*-Nährstoffabbaus von Grünlandaufwüchsen des Alpenraumes unterschiedlicher Vegetationsstadien sowie von Maissilagen und Heu – ein Beitrag zur Weiterentwicklung der Rationsgestaltung für Milchkühe. . Dissertation, Agrar- und Ernährungswiss. Fak., Christian-Albrechts-Universität, Kiel, Germany.

Kirchhof, S., Eisner, I., Gierus, M., Südekum, K.-H., 2010. Variation in the contents of crude protein fractions of different forage legumes during the spring growth. Grass Forage Sci. 65, 376-382.

Klopfenstein, T.J., Mass, R.A., Creighton, K.W., Patterson, H.H., 2001. Estimating forage protein degradation in the rumen. J. Anim. Sci. 79 (E. Suppl.), E208-E217.

Krawielitzki, K., Schmidt, T., Voigt, J., Kowalczyk, J., Gabel, M., 2006. Dynamics of microbial contamination of protein during ruminal *in situ* incubation of feedstuffs. J. Anim. Feed Sci. 15, 313-328.

Krishnamoorthy, U., Sniffen, C.J., Stern, M.D., Van Soest, P.J., 1983. Evaluation of a mathematical model of rumen digestion and an *in vitro* simulation of rumen proteolysis to estimate the rumen-undegraded nitrogen content of feedstuffs. Brit. J. Nutr. 50, 555-568.

Licitra, G., Hernandez, T., Van Soest, P., 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Technol. 57, 347-358.

Licitra, G., Lauria, F., Carpino, S., Schadt, I., Sniffen, C., Van Soest, P., 1998. Improvement of the *Streptomyces griceus* method for degradable protein in ruminant feed. Anim. Feed Sci. Technol. 72, 1-10.

Licitra, G., Van Soest, P., Schadt, I., Carpino, S., Sniffen, C., 1999. Influence of the concentration of the protease from *Streptomyces griseus* relative to ruminal protein degradability. Anim. Feed Sci. Technol. 77, 99-113.

Madsen, J., Hvelplund, T., 1994. Prediction of *in situ* protein degradability in the rumen. Results of a European ringtest. Live. Prod. Sci. 39, 201-212.

Mahadevan, S., Erfle, J.D., Sauer, F.D., 1980. Degradation of soluble and insoluble proteins by *Bacteroides amylophilus* protease and by rumen microorganisms. J. Anim. Sci. 50, 723-728.

Marquardt, D.W., 1963. An algorithm for least-squares estimation of nonlinear parameters. J. Soc. Ind. App. Math. 11, 431-441.

Mass, R.A., Lardy, G.P., Grant, R.J., Klopfenstein, T.J., 1999. *In situ* neutral detergent insoluble nitrogen as a method for measuring forage protein degradability. J. Anim. Sci. 77, 1565-1571.

Mathis, C.P., Cochran, R.C., Vanzant, E.S., Abdelgadir, I.E.O., Heldt, J.S., Olson, K.C., Johnson, D.E., Caton, J., Faulkner, D.B., Horn, G., Paisley, S., Mass, R., Moore, K., Halgerson, J., 2001. A collaborative study comparing an *in situ* protocol with single time-point enzyme assays for estimating ruminal protein degradability of different forages. Anim. Feed Sci. Technol. 93, 31-42.

McDonald, I., 1981. A revised model for the estimation of protein degradability in the rumen. J. Agric. Sci. 96, 251.

Michalet-Doreau, B., Ould-Bah, M.Y., 1992. In vitro and in sacco methods for the estimation of dietary nitrogen degradability in the rumen: a review. Anim. Feed Sci. Technol. 40, 57-86.

NRC, 2001. Nutrient Requirements of Dairy Cattle. 7th ed. National Academy Press, Washington, DC, USA.

SAS®, 2002. User's Guide: Statistics. Version 9.1. SAS Institute, Inc., Cary, NC, USA.

Shannak, S., Südekum, K.-H., Susenbeth, A., 2000. Estimating ruminal crude protein degradation with *in situ* and chemical fractionation procedures. Anim. Feed Sci. Technol. 85, 195-214.

Schwab, C.G., Huhtanen, P., Hunt, C.W., Hvelplund, T., 2005. Nitrogen requirements of cattle. In: Pfeffer, E., Hristov, A. (Eds.), Nitrogen and Phosphorus Nutrition of Cattle: Reducing the Environmental Impact of Cattle Operations, CABI Publishing, Wallingford, UK, pp. 13-70.

Sniffen, C.J., O'Connor, J.D., Van Soest, P.J., Fox, D.G., Russell, J.B., 1992. A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. J. Anim. Sci. 70, 3562-3577.

Stern, M.D., Bach, A., Calsamiglia, S., 1997. Alternative techniques for measuring nutrient digestion in ruminants. J. Anim. Sci. 75, 2256-2276.

Tamminga, S., Brandsma, G.G., Dijkstra, J., van Duinkerken, G., van Vuuren, A.M., Blok, M.C., 2007. Protein Evaluation for Ruminants: the DVE/OEB 2007 System, CVB documentation report nr. 53. CVB; Lelystad, The Netherlands.

Tedeschi, L.O., Pell, A.N., Fox, D.G., Llames, C.R., 2001. The amino acid profiles of the whole plant and four plant residues from temperate and tropical forages. J. Anim. Sci. 79, 525-532.

Universität Hohenheim – Dokumentationsstelle, (Ed.), 1997. DLG-Futterwerttabellen für Wiederkäuer, 7th ed. DLG-Verlag, Frankfurt/Main, Germany.

VDLUFA, 2007. Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten. Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik (VDLUFA-Methodenbuch), Bd. III. Die Chemische Untersuchung von Futtermitteln. VDLUFA-Verlag, Darmstadt, Germany.

Volden, H.E., 2011. NorFor-The Nordic feed evaluation system. Wageningen Academic Publishers, Wageningen, The Netherlands.

Wulf, M., Südekum, K.-H., 2005. Effects of chemically treated soybeans and expeller rapeseed meal on *in vivo* and *in situ* crude fat and crude protein disappearance from the rumen. Anim. Feed Sci. Technol. 118, 215-227.

Yu, P., Christensen, D.A., McKinnon, J.J., Markert, J.D., 2003. Effect of variety and maturity stage on chemical composition, carbohydrate and protein subfractions, *in vitro* rumen degradability and energy values of timothy and alfalfa. Can. J. Anim. Sci. 83, 279-290.

Zinn, R.A., Owens, F.N., 1986. A rapid procedure for purine measurement and its use for estimating net ruminal protein synthesis. Can. J. Anim. Sci. 66, 157-166.

CHAPTER 4

Estimating utilisable crude protein at the duodenum, a precursor to metabolisable protein for ruminants, from forages using a modified gas test

B. Edmunds[1,2], K.-H. Südekum[1*], H. Spiekers[2], M. Schuster[3], F.J. Schwarz[4]

[1]*Institute of Animal Science, University of Bonn, Endenicher Allee 15, 53115 Bonn, Germany*

[2]*Institute for Animal Nutrition and Feed Management, Bavarian State Research Centre for Agriculture (LfL), Prof.-Dürrwaechter-Platz 3, 85586 Poing, Germany*

[3]*Department for Quality Assurance and Analysis (Central Laboratory,) Bavarian State Research Centre for Agriculture (LfL), Prof.-Zorn-Str. 20 c, 85586 Poing, Deutschland*

[4]*Animal Nutrition Weihenstephan, Technical University of Munich, Weihenstephaner Berg 3, 85350 Freising, Germany*

Submitted for publication

Abstract

As protein evaluation systems are evolving, they are increasing in their sophistication and complexity. In almost all systems estimates of microbial crude protein (MCP) and ruminally undegraded feed CP (RUP) must be determined. The problem lies mainly in the accuracy of these measurements, especially RUP, which is often estimated by the controversial *in situ* technique. A new *in vitro* method has been developed which provides a direct estimate of combined MCP and RUP. The modified Hohenheim gas test (modHGT) involves incubation of a feedstuff in rumen fluid. The non-ammonia N content after incubation is used to determine utilisable crude protein at the duodenum (uCP) which is defined as the sum of MCP and RUP at the duodenum. In this study 23 forages were tested using the modHGT and presented at three assumed rates of rumen passage (K_p 0.02, 0.04 and 0.06 h^{-1}). The results were regressed against uCP values calculated using the standard procedure in Germany. Calculated uCP correlated significantly with determined uCP at all rates of passage (K_p2: $P<0.038$, $r^2 = 0.19$; K_p4: $P<0.0001$, $r^2 = 0.56$; K_p6: $P<0.0001$, $r^2 = 0.67$). Due to the simplicity of the reference method it is possible that the modHGT provides more accurate results. Although the new method is also simple, it considers interactions between carbohydrate and protein degradation by rumen microbes and uCP is estimated from the fermentation end product, ammonia crude protein may then be used to calculate metabolisable protein.. Utilisable

Keywords: Rumen, Forage, Protein value, Metabolisable protein, Methods

Introduction

Balanced dairy systems should meet the amino acid requirement of cows but not exceed it. Excess N is harmful to the environment and costly to the farmer. But meeting the amino acid requirement of dairy cows is a complicated and challenging goal. Worldwide, protein evaluation systems are constantly being updated and are increasing in their sophistication. They are also becoming very complex.

Protein evaluation systems should accurately quantify supply of microbial crude protein (MCP) and rumen undegraded CP (RUP) at the duodenum as well the N and amino acid requirements, respectively, of the rumen microbes and the host animal. Most systems attempt this, although methods and assumptions differ in the estimation of these variables. For example, estimating MCP usually requires an estimate of energy available for microbial fermentation and growth. However, the definition of rumen available energy still varies between systems; the French PDI system (INRA, 1989) uses fermentable organic matter, total digestible nutrients is used in the NRC system (NRC, 2001), digestible organic matter minus RUP in Finnish system (MTT, 2010) and fermentable organic matter in the rumen in the Dutch system (Tamminga et al., 2007; Van Duinkerken et al., 2010). Estimating microbial efficiency also varies; particularly as using a constant to describe microbial efficiency is being abandoned for more complex equations in newer systems. The Dutch, British (FiM: Thomas, 2004) and the new NorFor (Volden et al., 2011; Denmark, Norway, Sweden and Iceland) systems calculate microbial efficiency from fractional rumen outflow rates (K_p). However, in the Dutch system this is substrate specific whilst the British and NorFor systems use fractional outflow rates based on dry matter intake. The Cornell Net Carbohydrate and Protein System (CNCPS: Fox et al., 2004) is different again in that it assumes efficiency is related to fractional degradation rate.

Another highly complex variable is RUP and large differences in its estimation exist among systems. Most systems derive degradability estimates from the *in situ* procedure, a method which is subject to large amount of error and variation (Nocek and Russell, 1988; Michalet-Doreau and Ould-Bah, 1992; Hvelplund and Weisbjerg, 2000; Schwab et al., 2005). Ongoing problems still occur in the repeatability of the *in situ* procedure, even where attempted standardisation has been made (Madsen and Hvelplund, 1994; Mathis et al., 2001). In fact, using a constant to represent protein degradability has been shown to be just as accurate as using *in situ* determined, feed-specific RUP values (Touri et al., 1998; Schwab et al., 2005). The point is that the measurement, calculation and assumptions used in estimating

the large and increasing number of variables in modern protein evaluation systems are all subject to error and accumulated error could actually decrease the robustness and thus accuracy of the final result: the protein value of the feed.

In vitro procedures offer alternatives to animal-dependent experiments using *in situ* or *in vivo* methods. In a companion paper (Edmunds et al., 2012; Chapter 3) two *in vitro* methods were analysed for their accuracy in estimating RUP. This paper presents a new, simple, substrate-specific, and labour-efficient *in vitro* method of analysing feed protein. The method bypasses the need to estimate RUP altogether. The modified Hohenheim gas test (modHGT) was developed by Steingaß et al. (2001) and applies a modification (Raab et al., 1983) to the standard Hohenheim gas test (Menke and Steingaß, 1988) whereby ammonia is measured after incubation with rumen fluid. The non-ammonia-N concentration at the end of the incubation forms the basis in calculating utilisable CP at the duodenum (uCP), which is defined at the sum of MCP and RUP at the duodenum. The procedure also shows potential for calculating 'effective uCP' to represent selected rates of ruminal passage, which would provide a more suitable uCP value for animals fed at various levels. The present study focused on assessing the validity of this new method for its potential use in routine forage analysis. The current German system (GfE, 2001), with the variable RUP estimated using the *in situ* procedure, was used to validated the procedure. It was expected that the modHGT would estimate uCP as well as the GfE system but with a higher sensitivity to the nutritional characteristics of individual feeds.

Materials and Methods

Feedstuffs

Twenty three forage samples from harvest year 2008 were selected to cover a variety of conservation types. Samples included fresh (n = 12: white clover, lucerne, perennial ryegrass, ryegrass/white clover mixed sward and meadow grass), and conserved forage: hay (n = 1: meadow grass), silage (n = 6: meadow grass wilted to 350, 500 and 650 g DM at a fast or slow rate of moisture loss) and artificially dried (n = 4: meadow grass and lucerne). A detailed description of the samples can be read in Chapter 3, Table 1.

General analysis

All feeds were freeze-dried and milled through a 3 mm screen for the *in situ* trial and a 1 mm screen for all other analyses. A complete description of analyses of proximate variables can be read in Chapter 3. Metabolisable energy was calculated according to GfE (2008):

ME (MJ/kg DM) = 7.81 + 0.07559 GP - 0.00384 Ash + 0.00565 CP + 0.01898 CL - 0.00831 ADFom

where GP is *in vitro* gas production at 24 hours (ml/200 mg DM), CL is crude lipids (g/kg DM), ADFom is acid detergent fibre expressed without residual ash (g/kg DM) and Ash and CP are expressed in g/kg DM.

In situ procedure

Methods describing the *in situ* trial can be read in detail in Chapter 3. Briefly, the procedure followed basic guidelines of Madsen and Hvelplund (1994) with incubation periods of 2, 4, 8, 16, 24, 48 and 96 hours and using three non-lactating cows per feedstuff. Water soluble material was estimated by mixing duplicate samples in 100 ml, 40°C distilled water and then filtered through No. $595^{1/2}$, diameter 270 mm filter paper (Schleicher and Schuell, Dassel, Germany). The equation of Hvelplund and Weisbjerg (2000) was used to correct CP disappearance for small particle loss at each incubation time point. Correction for microbial attachment to undegraded feed particles was carried out according to Edmunds et al. (2012; Chapter 3). Effective degradability of CP (g/kg CP) at assumed passage rates of 0.02, 0.04 and 0.06 h^{-1} (K_p2, 4, 6) was calculated according to McDonald (1981), but with the assumption that no degradation occurred during the lag phase (Wulf and Südekum, 2005). Finally, RUP (g/kg CP) was calculated as 1000-effective degradabilty.

Modified Hohenheim gas test

The modHGT (Steingaß et al., 2001) was developed with the aim of directly measuring ammonia N following incubation of a feedstuff in a rumen fluid/buffer solution. The non-ammonia-N multiplied by 6.25 represents uCP. The modHGT follows procedures of the regular HGT (Menke and Steingaß, 1988) with a chemical alteration of 2 g/l increase in NH_4HCO_3 and 2 g/l decrease in $NaHCO_3$ in the buffer solution. This modification prevents N from becoming a limiting factor. Recommended incubation times are 8 and 24 h for concentrates and 8 and 48 h for forages (Leberl et al., 2007). Incubation for 24 h is unsuitable for forages (Edmunds, unpublished). Rumen fluid from cows or sheep may be used.

In the present study rumen fluid was collected from two to three fistulated sheep receiving a 50:50 grass hay:pelleted compound maintenance ration twice daily in unequal proportions: one third at 07:00 and two thirds at 15:30 h. The fluid was extracted before the morning feed and transported in a pre-warmed thermos, which was completely filled, and immediately sealed. The rumen fluid was filtered through two layers of cheese cloth into a warm flask and

then added to the reduced buffer solution. After allowing 15 minutes to acclimatise, 30 ml of the solution was added to a pre-warmed syringe containing 200 ± 30 mg substrate. Syringes were immediately placed in a rotary incubator which had been pre-warmed to 39°C. The starting time of the incubation was recorded after all syringes had been filled. Each feedstuff was analysed in duplicate and over two runs i.e. two different batches of rumen fluid, which were considered as biological replicates. At the end of each incubation time (8 and 48 h) gas volume was recorded and syringes put on ice to stop microbial activity. Gas production (GP) was also recorded at 24 h for use in the calculation of ME. At both the 8 and 24 h readings the plunger was set back to 30 ml (not done for the blank). A blank, containing rumen fluid/buffer solution without added substrate (NH_3N_{blank}), was also incubated in duplicate alongside the samples i.e. for 8 and 48 h. Ammonia-N (mg NH_3-N/30 ml) from both the blank and from the syringes containing substrate (NH_3N_{sample}) was measured by distillation (Vapodest 50s carousel; Gerhardt, Königswinter, Germany) and used in the following calculation (H. Steingaß, unpublished):

$$uCP\ (g/kg\ DM) = ((NH_3N_{blank} + N_{sample} - NH_3N_{sample})/weight\ (mg\ DM)) \times 6.25 \times 1000$$

where N_{sample} is N added to the syringe from the measured amount of feedstuff (mg), weight is the amount of sample weighed into the syringe and calculated to DM and other variables are as previously described.

When using a live product such as rumen fluid small biological fluctuations between runs are inevitable. To correct for this a protein standard (provided by the University of Hohenheim) was analysed with every run. The standard was a concentrate mix of (per kg DM) 450 g rapeseed meal, 300 g faba beans and 250 g molassed sugar beet pulp, and had a CP content of 225 g/kg DM. The correction follows the same method as for gas production (Menke and Steingaß, 1988) whereby the mean uCP value for the standard (at 8, 24 or 48 h) is divided by the recorded value of the standard for that run and all other samples are multiplied by the resulting correction factor. Runs were repeated if the correction factor, for either incubation time, lay outside the range of 0.9 to 1.1. The hay and concentrate standards typically used for correcting gas production were also included in the incubation not only to correct gas production values, but to ensure the rumen fluid solution followed typical fermentation.

An attempt was made to calculate effective uCP. Like effective protein degradability, effective uCP should represent various rates of solid flow through the rumen i.e. passage rate.

Following correction using the protein standard, uCP values from the two incubation time points of one run were plotted against a log time (ln(t)) scale, where 't' is the time of incubation, and the resulting regression equation was used to calculate effective uCP to assumed passage rates (K_p) of 0.02, 0.04 and 0.06 h^{-1} using the formula:

effective uCP = y + a x ln(1/K_p)

where *y* is the intercept and *a* is the slope. Between-run regression equations will differ slightly due to methodological error, however variations to the slope and intercept balance out to provide effective uCP values that can be used as repeats (see Table 2). Effective uCP should only be calculated if the standard samples are within the range of 0.9 to 1.1 i.e. they differ by ≤ 10% from their average value. The assumption of a linear decrease in uCP with ln time was demonstrated using soybean meal incubated at several time points spanning 4 to 48 h (H. Steingaß, unpublished). Forages do not follow this linearity as closely as concentrates and uCP after 24 hours incubation is often higher than or equal to uCP at 8 hours (Edmunds, unpublished). The legitimacy of this calculation will be discussed later in the paper.

Calculations and statistics

Reference values, against which results of the modified HGT were validated, were calculated from one of 12 equations defined by Lebzien and Voigt (1999) and adopted by the GfE (2001). The chosen equation was specific for feedstuffs containing less than or equal to 70 g crude lipids/kg DM and using ME rather than digestible organic matter, as a measure of available energy:

uCP (g/kg DM) = [11.93 - (6.82 x (RUP/CP))] x ME + 1.03 x RUP

where RUP and CP are in g/kg DM and ME is in MJ/kg DM. Effective RUP at K_p 0.02, 0.04 and 0.06 h^{-1}, estimated by *in situ* analysis, was the only alteration in providing the three calculated uCP values for each sample. These values were used to represent the three chosen rates of passage. Utilisable CP determined by the modHGT (independent variable) was regressed against calculated values (dependent variable) using the PROC REG procedure of SAS (version 9.1). Relationships were deemed significant at $P<0.05$. Relationships are described using the coefficient of determination (r^2), and root mean square error (RMSE), which describes the standard deviation of the error, and regression coefficients (slope, intercept). Predicted values (dependent variable) were regressed against residuals to determine the presence or absence of linear bias.

Results

Results of the proximate and *in situ* analyses are presented in detail in a companion paper (Edmunds et al., 2012; Chapter 3) and Table 1 presents a quick overview of the mean, standard deviation and range in proximate variables. Twenty five forage samples were analysed in the companion paper, however insufficient material remained from the two highest moisture silage samples for subsequent analyses. Thus only 23 forages have been presented in this paper.

Table 1 Mean, standard deviation (SD) and range of dry matter (DM), crude protein (CP), ash, crude lipids (CL), neutral detergent fibre (NDF: assayed without heat stable amylase) and acid detergent fibre (ADF) both presented inclusive of residual ash, and metabolisable energy (ME) of the 23 analysed forages.

	DM	CP	Ash	CL	NDF	ADF	ME
	g/kg	----------g/kg DM----------					MJ/kg DM
Mean	424.1	180.6	97.4	31.8	398.9	257.4	10.6
SD.	277.0	35.3	14.8	13.0	57.4	35.4	0.7
Maximum	900.0	276.0	116.6	59.0	513.3	329.8	11.5
Minimum	135.0	109.0	64.0	14.8	226.6	197.8	9.2

Results from the standard protein sample demonstrate the high between run repeatability of the modHGT. Table 2 presents data from 5 runs. The expected values of the protein standard were 232 and 97 for incubation times 8 and 48 h, respectively. The recorded averages were 221 ± 6 and 102 ± 12 for 8 and 48 h, respectively. A slightly different average is expected due to inter-laboratory variations. One of the purposes of the standard is to reduce this variation. The correction factor was within range for all runs except run 3, where the 48 h value deviated by more than 10% from the expected value. This run, therefore, was not used in the analysis and all samples within this run were repeated. The slope and intercept used for calculating effective uCP are also presented along with effective uCP at the three assumed passage rates (Table2). From this data it is clear that seemingly large between-run variation of

the slope and intercept do not translate into similar variation in the effective uCP values. In fact, the between-run repeatability is remarkable good. To further demonstrate this, corrected uCP values of all samples from each incubation time of two runs were regressed against each other (n = 23). The coefficient of determination for 8 and 48 hours was 0.90 and 0.77, respectively. The average difference in uCP (run 1 - run 2) for 8 and 48 hours was 1.24 ± 8.92 and 3.31 ± 6.46 g/kg DM, respectively. This high correlation carried over to effective uCP with r^2 of 0.76, 0.96 and 0.93 for K_p2, 4 and 6, respectively. Generally, repeatability was slightly higher at the shorter incubation time.

Table 2 Between run variation shown by the standard protein sample where uCP is uncorrected utilisable crude protein after 8 or 48 h incubation, correction factor is the proportional variation of uCP to the expected value of the standard, slope and intercept originate from regression of uCP against log (time) within a run and effective uCP (g/kg DM) is uCP presented at three assumed rates of passage.

Run	Time	uCP	Correction	Slope	Intercept	Effective uCP		
	h	g/kg DM	factor		g/kg DM	K_p2	K_p4	K_p6
1	8	220	1.06	-71.17	367.80	89	139	168
	48	92	1.05					
2	8	220	1.06	-64.56	353.85	101	146	172
	48	104	0.93					
3	8	212	1.09	-50.70	317.71	119	155	175
	48	121	0.80					
4	8	229	1.01	-76.65	388.86	89	142	173
	48	92	1.05					
5	8	222	1.04	-68.94	365.49	96	144	172
	48	99	0.98					

Table 3 presents CP, ME, RUP (K_p4) and effective uCP (calculated: GfE, 2001 and determined: modHGT) on a per feedstuff basis. The modHGT results are presented as a difference from their respective calculated value (calculated - determined). Determined uCP was generally lower than calculated uCP at K_p2 and higher at K_p6. Based on the differences between the mean for each method and the RMSE (Table 4), calculated uCP was best

predicted at K_p4. The largest between-method difference at K_p4 was from the first-cut, artificially dried lucerne sample with determined uCP being 26 g/kg DM higher than calculated uCP. This sample also had an unusually high CP concentration (206 g/kg DM) compared to its fresh counterpart (185 g/kg DM). Reanalysis of CP using the Kjeldahl method reduced the difference in CP between the two samples to only 5 g/kg DM (177 and 182 g CP in fresh and dried material, respectively). Recalculation of uCP at K_p4 reduced the difference from the calculated value to 14 g/kg DM. The original uCP value of this sample has been retained in the regression analysis as it was not out of range of the expected values. It should be noted, however, that this sample does slightly reduce the coefficient of determination.

Results of the linear regression analysis between calculated and determined uCP at the three assumed rates of passage are presented in Table 4. There was a significant correlation at all rates of passage, though this was only weak at K_p2 ($P=0.038$) and the r^2 was low (0.19). The strength of the relationship improved with increasing passage rate (K_p4: $r^2 = 0.56$, $P<0.0001$; K_p6: $r^2 = 0.67$, $P<0.0001$). Slope, intercept and RMSE were similar for K_p4 and 6. Analysis of the residuals against predicted values revealed no linear bias at any of the three passage rates ($P>0.05$). Division of the data into forage types (fresh, ensiled, dried) presented a better picture of the origins of the variation (Figure 1; K_p4).

Table 3 Calculated (GfE) and determined (modHGT) utilisable crude protein (uCP; g/kg DM) presented at assumed passage rates of 0.02, 0.04 and 0.06 h^{-1}. Utilisable CP from modHGT is expressed as a difference (g/kg DM) from the calculated value (GfE - modHGT). Crude protein (CP; g/kg DM) and ruminally undegraded dietary CP ($RUP_{0.04}$; g/kg CP) are also presented.

				-----$uCP_{0.02}$-----		-----$uCP_{0.04}$-----		-----$uCP_{0.06}$-----	
Feedstuff	Harvest	CP	RUP	GfE	modHGT	GfE	modHGT	GfE	modHGT
Fresh									
Perennial ryegrass	1	109	200	141	-31	143	-4	144	12
Perennial ryegrass	3	148	222	131	-12	138	13	143	27
White clover	1	241	175	152	-29	162	6	171	23
White clover	3	276	192	159	-19	172	11	183	24
Ryegrass/white clover	1	163	180	139	-19	144	7	149	21
Ryegrass/white clover	1	148	145	131	-16	134	11	137	26
Meadow grass 1	1	140	208	137	-29	141	-6	145	6
Meadow grass 1	2	149	222	130	-10	136	9	140	19
Meadow grass 2	1	184	168	148	-22	154	9	160	25
Meadow grass 2	1	193	162	149	-21	156	8	162	23
Lucerne	1	185	129	127	-25	131	5	135	22
Lucerne	3	191	148	128	-15	133	14	137	29
Grass Silage[a]									
Fast 350	2	189	142	137	-34	141	-4	145	13
Slow 350	2	191	123	133	-32	137	-11	140	1
Fast 500	2	186	152	135	-27	140	-2	145	11
Slow 500	2	195	129	136	-34	140	-9	143	5
Fast 650	2	179	230	138	-44	147	-13	154	3
Slow 650	2	191	189	137	-34	145	-10	151	2
Dried									
Meadow grass 1-hay	1	128	269	136	-36	141	-15	145	-4
Meadow grass 2	1	190	230	154	-45	164	-1	172	23
Meadow grass 2	1	194	225	155	-46	165	1	173	26
Lucerne	1	206	210	133	-7	142	26	150	43
Lucerne	3	178	255	134	-24	141	9	148	26

[a] Fast and slow refer to the rate of moisture loss during wilting, the number refers to DM content (g/kg).

Table 4 Results of linear regression analysis between calculated (GfE, 2001) and determined (modHGT) uCP (mean and standard deviation (SD)) from 23 forages. The r^2, slope, intercept, root mean squared error (RMSE) and P values of intercept and the model are presented at three assumed rates of passage (K_p).

	K_p h^{-1}	uCP g/kg DM	SD	Slope	Intercept	$P_{intercept}$	RMSE	r^2	P
GfE	0.02	139.0	8.6	0.36	96.50	<0.0001	8.38	0.19	0.038
modHGT		112.7	11.3						
GfE	0.04	145.3	10.6	0.54	65.29	<0.001	7.59	0.56	<0.0001
modHGT		148.0	15.5						
GfE	0.06	150.4	12.4	0.56	56.97	<0.001	7.64	0.67	<0.0001
modHGT		168.6	19.3						

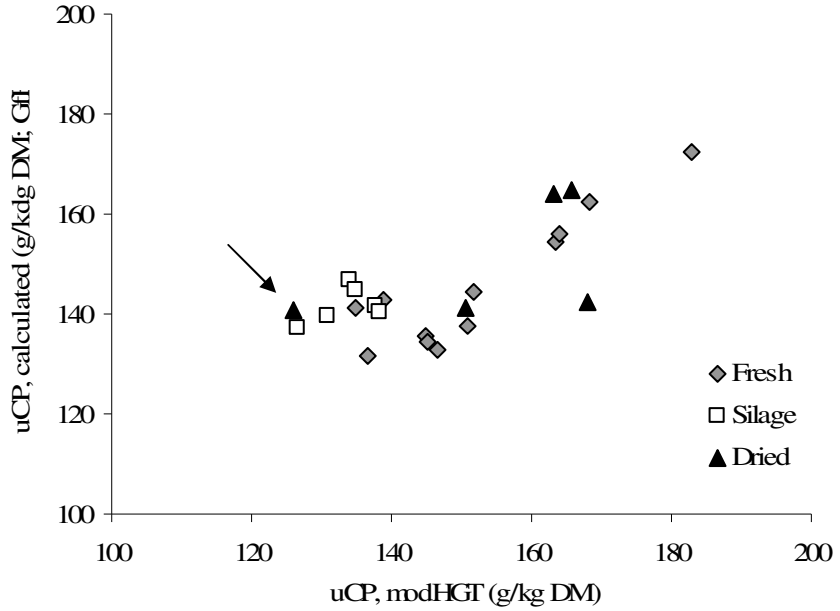


Figure 1 Correlation between calculated (GfE, 2001: Y-axis) and determined (modHGT: X-axis) utilisable crude protein at the duodenum (uCP, g/kg DM) of different forage types at passage rate 0.04 h^{-1}. The point highlighted with an arrow represents the hay sample.

Discussion

The significant correlation between calculated and determined uCP at all assumed rates of passage indicates that the modHGT displays accuracy in ranking feeds according to protein value. A similar experiment using 10 rapeseed meal and 7 soybean meal samples and incubation times of 8 and 24 h gave similar results at a passage rate of 0.05 h^{-1} (r^2 = 0.68, P<0.0001; Nibbe et al., 2001; Südekum et al., 2003), demonstrating that the method is also suitable for concentrates. To improve understanding of the accuracy of the modHGT, averaged uCP results (K_p4) for fresh forage (n = 12) and silage (n = 6) from the present study were compared to tabulated DVE averages. By definition, DVE is intestinally digestible protein and is the Dutch equivalent of metabolisable protein. Tabulated averages of RUP and MCP for fresh forage (n = 65) and grass silage (n = 500: Tamminga et al., 2007) were summed to produce DVE values at an assumed passage rate of 0.045 h^{-1}. Utilisable CP determined from the modHGT were converted to metabolisable protein using the constants 0.73 and 0.85, which represent the proportion of amino acid-N in duodenal non-ammonia-N and the absorption coefficient of amino acid-N, respectively (GfE, 2001). The results were remarkably similar. For fresh forage and silage respectively, metabolisable protein (g/kg DM) was 94.5 and 82.9 for the modHGT system and 98.6 and 82.7 for the Dutch system. This is a clear demonstration that the modHGT provides realistic estimates of metabolisable protein.

One might point out that the coefficients of determination from the results of the present study are not very strong. This can mainly be explained by the limitations of the GfE (2001) equation. The equation was derived from results of *in vivo* trials conducted at various feeding levels using mixed rations. No doubt this will cause discrepancies when analysing individual feeds and calculating them to represent varying rates of passage. Furthermore, the only factor that changes in the calculation of the three GfE values is effective RUP. Consideration of the effect of passage rate on RUP alone severely limits the accuracy of such an equation in predicting effective uCP and the effect of passage rate on MCP synthesis should also be considered. Additionally, RUP was calculated using the *in situ* technique, which is subject to high levels of variation, adding further error to the calculated values. The values used in this study are, however, associated with a high level of confidence due to the use of highly standardised methods and correction for microbial attachment (Edmunds et al., 2012; Chapter 3). Moreover, replacing *in situ* RUP with tabulated RUP did not improve the results. For these reasons it is likely that the modHGT provides a more accurate representation of uCP at the

chosen rate of passage. Stronger regression statistics between GfE (2001) and modHGT values were not and should not be expected.

The fact is, it is difficult to ascertain just how accurate the results are as no good reference method exists. *In vivo* measurements are generally accepted as the 'gold standard'. However, this technique requires access to ruminally and duodenally canulated animals and is also subject to a certain level of error, mainly due to the use of markers and variation between animals (Stern et al., 1997). Additionally, due to the high labour input and time taken to run the experiment, only a small number of samples can be analysed using *in vivo* techniques. The problem concerning generation of accurate reference values against which to validate new methods is ongoing. Despite the previously described problems, the reference values used in this study were calculated using a method that has been proven to be robust and accurate in predicting metabolisable protein and milk protein yield. Schwab et al. (2005) compared the accuracy of predicting of milk protein yield from metabolisable protein between six protein evaluation systems (British: AFRC, 1992; French: INRA, 1989; USA: NRC, 2001; Danish: Madsen et al., 1995; Finnish: Tuori et al., 1998; German: GfE, 2001) using Finnish data sets. To predict uCP the simplest GfE equation was chosen, which required knowledge of only ME and CP. Using the previously described constants (0.73 and 0.85: GfE, 2001) uCP was converted to metabolisable protein. Despite its simplicity, the German system performed as well as other systems, even though it used a constant for rumen CP degradability for all feedstuffs. It is well known that rumen degradability differs among feeds so why the German system performed so well is not entirely clear. Touri et al. (1998) also observed improved prediction of milk protein yield using a constant degradability rather than *in situ* determined protein degradability. It could be supposed that these results reflect the inaccuracy of effective degradability measured by ruminal *in situ* incubation (Schwab et al., 2005; Huhtanen and Hristov, 2009; Huhtanen, 2010).

In the pursuit of accuracy protein evaluation systems must be improved. Although robust, one of the main limitations of the current GfE method is that it is not very sensitive to the composition of individual feedstuffs. In the previously mentioned study by Schwab et al. (2005) metabolisable protein estimated from the GfE system had a comparatively low between-sample standard deviation. This was attributed to the simplicity of the model and its failure to incorporate many of the factors affecting flow of MCP and RUP to the small intestine. Despite inclusion of RUP in the equation used in the present study, the model is still not sophisticated enough to accurately describe changes to uCP on a per sample basis. In this

respect, the modHGT is highly advantaged. As clearly demonstrated in Table 4, at all assumed rates of passage the between sample standard deviation is higher from modHGT. If interactions between the rate and extent of carbohydrate and protein degradation are assumed to occur naturally during incubation in rumen fluid, then the method can be assumed to be highly sensitive to degradation characteristics of individual feedstuffs. Another advantage of the method is that mixed rations may also be analysed, providing a possibly more accurate result than the additive value calculated from analysis of individual feed components.

There are, of course, limitations to this *in vitro* method. Firstly, it is a closed system and therefore does not consider the effect of continual rumen flow and the introduction of new carbohydrates to the rumen through feeding and the subsequent effect on the microbial population. Nitrogen recycling is also not considered; although the method has been developed so that N is not limiting therefore any effects of N recycling would be minimal and probably insignificant. In analysing low CP feeds a further increase of N in the buffer solution may be considered. Secondly, adoption of fractional passage flow rates in other systems describing the passage of solid (based on forage or concentrate) and liquid pools would imply that the method of calculation to passage rate described in this study may be too simple. The legitimacy of the mathematics used to calculate effective uCP may also be debated. Nevertheless is it a step forward from the current method which uses singular uCP values for animal at all levels of production. As of yet it is unclear how to combine uCP values with knowledge on fractional passage rate and whether or not it would improve the accuracy of the results. More work is required in this area. Thirdly, correction of forage uCP data through use of a concentrate standard may be further improved through use of a forage-based standard. Components of concentrates and forages are metabolised by different bacteria (Russell et al., 1992) thus small weekly fluctuations in initial microbial composition may affect metabolism patterns of feed types, namely concentrates and forages, in different ways. Additionally, the type of substrate and the rate of appearance of fermentation end products may also affect the microbial population during incubation. A concentrate would favour growth of non-structural carbohydrate-fermenting bacteria, which can produce ammonia and use ammonia, amino acids or peptides as an N source. Forage would favour structural carbohydrate-fermenting bacteria, which do not ferment peptides or amino acids and only use ammonia as an N source (Russell et al., 1992). The point is that weekly fluctuations in fermentation characteristics of a concentrate may not reflect that of forages. Therefore it may make more sense to use a forage-based standard when analysing forages.

Conclusions

The new modified gas test shows high potential for use as an accurate method of estimating uCP. The procedure is simple, has a high level of repeatability, and requires knowledge only of CP prior to incubation. Validation with *in vivo* data is recommended. Incorporation of uCP into protein evaluation systems could be a progressive step in simplification and subsequent reduction in error as it gives a direct estimate of the sum of MCP and RUP at the duodenum. Conversion to metabolisable protein should employ the same knowledge and assumptions on endogenous CP, amino acid content and digestibility as already used in current systems.

Acknowledgments

This study was financially supported by the Bavarian Ministry for Agriculture and the Institute of Animal Science, University of Bonn. Thanks go out to Laboratory staff at the LfL-Bayern for the proximate analysis, Dr. H. Nußbaum (LAZBW-Aulendorf) for providing the silages; Dr. J. Hummel, T. Priambodo, stable staff and N. Wahl (University of Bonn) for assistance with the modHGT, collection of rumen fluid and ammonia analysis; Dr. K. Rutzmoser for his assistance with SAS; P. Rauch for assistance in sample collection.

References

AFRC, 1992. Technical Committee on Responses to Nutrients, Report No. 9. Nutritive requirements of ruminant animals: protein. Nutr. Abstr. Rev., Ser. B 62, 787-835.

Edmunds, B., Südekum, K.-H., Spiekers, H., Schwarz, F., 2012. Estimating ruminal crude protein degradation of forages using in situ and in vitro techniques. Submitted for publication (ANIFEE-11-3541R1).

Fox, D.G., Tedeschi, L.O., Tylutki, T.P., Russell, J.B., Van Amburgh, M.E., Chase, L.E., Pell, A.N., Overton, T.R., 2004. The Cornell net carbohydrate and protein system model for evaluating herd nutrition and nutrient excretion. Anim. Feed Sci. Technol. 112, 29-78.

GfE, 2001. Empfehlungen zur Energie- und Nährstoffversorgung der Milchkühe und Aufzuchtrinder. DLG-Verlag, Frankfurt/Main, Germany.

GfE, 2008. New equations for predicting metabolisable energy of grass and maize products for ruminants. Proc. Soc. Nutr. Physiol. 17, 191-197.

Huhtanen, P., 2010. Evaluation of protein value of forages, In: Jambor, V.J., S.;Vosynková, P.; Procházka, P.; Vosynková, D.; Kumprechtová, D. (Eds.), 14th Int. Symp. Forage Conservation. Brno, Czech Republic, 58-74.

Huhtanen, P., Hristov, A.N., 2009. A meta-analysis of the effects of dietary protein concentration and degradability on milk protein yield and milk N efficiency in dairy cows. J. Dairy Sci. 92, 3222-3232.

Hvelplund, T., Weisbjerg, M., 2000. In situ techniques for the estimation of protein degradability, In: Givens, D., Owen, E., Axford, R.F.E., Omed, H.M. (Eds.), Forage Evaluation in Ruminant Nutrition, CAB International, Wallinford, UK, pp. 233-258.

INRA, 1989. Ruminant Nutrition: Recommended Allowances and Feed Tables. John Libbey Eurotext, Paris, France.

Krawielitzki, K., Schmidt, T., Voigt, J., Kowalczyk, J., Gabel, M., 2006. Dynamics of microbial contamination of protein during ruminal in situ incubation of feedstuffs. J. Anim. Feed Sci. 15, 313-328.

Leberl, P., Gruber, L., Steingaß, H., Schenkel, H., 2007. Comparison of the methods modified Hohenheimer Futterwerttest (moHFT) and Cornell system for determination of nxp-content of concentrates. In: Kapun, S., Kramberger, B., Ceh, T. (Eds.) 16th International Science Symposium on Nutrition of Domestic Animals, Radenci, Slovenia, pp. 171-176.

Lebzien, P., Voigt, J., 1999. Calculation of utilisable crude protein at the duodenum of cattle by two different approaches. Arch. Anim. Nutr. 52, 363-369.

Madsen, J., Hvelplund, T., 1994. Prediction of in situ protein degradability in the rumen. Results of a European ringtest. Live. Prod. Sci. 39, 201-212.

Madsen, J., Hvelplund, T., Weisbjerg, M.R., Bertilsson, J., Olsson, I., Spörndly, R., Harstad, O.M., Volden, H., Touri, M., Varvikko, T., Huhtanen, P., Olafsson, B.I., 1995. The AAT/PBV protein evaluation system for ruminants. A revision. Norw. J. Agric. Sci. Suppl. 19, 1-37.

Mathis, C.P., Cochran, R.C., Vanzant, E.S., Abdelgadir, I.E.O., Heldt, J.S., Olson, K.C., Johnson, D.E., Caton, J., Faulkner, D.B., Horn, G., Paisley, S., Mass, R., Moore, K., Halgerson, J., 2001. A collaborative study comparing an in situ protocol with single time-point enzyme assays for estimating ruminal protein degradability of different forages. Anim. Feed Sci. Technol. 93, 31-42.

McDonald, I., 1981. A revised model for the estimation of protein degradability in the rumen. J. Agric. Sci. 96, 251.

Menke, K.H., Steingaß, H., 1988. Estimation of the energetic feed value from chemical analysis and in vitro gas production using rumen fluid. Anim. Res. Dev. 28, 7-55.

Michalet-Doreau, B., Ould-Bah, M.Y., 1992. In vitro and in sacco methods for the estimation of dietary nitrogen degradability in the rumen: a review. Anim. Feed Sci. Technol. 40, 57-86.

MTT, 2010. Rehutaulukot ja ruokintasuositukset (Feed Tables and Feeding Recommendations), Agrifood Research Finland, www.agronet.fi/rehutaulukot, Feb. 2011.

Nibbe, D., Lebzien, P., Spiekers, H., Steingaß, H., Südekum, K.-H., 2001. Vergleich verschiedener *in vitro*- und *in situ*-Verfahren zur Beurteilung des Proteinwertes von Raps- und Sojaextraktionsschrot. 113. VDLUFA-Kongress, Berlin, Kurzfassungen der Vorträge 111 (Abstr.).

Nocek, J.E., Russell, J.B., 1988. Protein and energy as an integrated system. Relationship of ruminal protein and carbohydrate availability to microbial synthesis and milk production. J. Dairy Sci. 71, 2070-2107.

NRC, 2001. Nutrient Requirements of Dairy Cattle. 7th ed. National Academy Press, Washington, DC, USA.

Raab, L., Cafantaris, B., Jilg, T., Menke, K.H., 1983. Rumen protein degradation and biosynthesis. 1. A new method for determination of protein degradation in rumen fluid in vitro. Br. J. Nutr., 569-582.

Russell, J.B., O'Connor, J.D., Fox, D.G., Van Soest, P.J., Sniffen, C.J., 1992. A net carbohydrate and protein system for evaluating cattle diets: 1. Ruminal fermentation. J. Anim. Sci. 70, 3551-3561.

SAS, 2002. User's guide: Statistics, SAS Institute, Inc., Cary, NC. USA.

Schwab, C.G., Huhtanen, P., Hunt, C.W., Hvelplund, T., 2005. Nitrogen requirements of cattle, In: Pfeffer, E., Hristov, A. (Eds.), Nitrogen and Phosphorus Nutrition of Cattle: Reducing the Environmental Impact of Cattle Operations, CABI Publishing, Wallingford, UK, pp. 13-70.

Steingaß, H., Nibbe, D., Südekum, K.-H., Lebzien, P., Spiekers, H., 2001. Schätzung des nXP-Gehaltes mit Hilfe des modifizierten Hohenheimer Futterwerttests und dessen

Anwendung zur Bewertung von Raps- und Sojaextraktionsschroten. 113. VDLUFA-Kongress, Berlin, Kurzfassungen der Vorträge 114 (Abstr.).

Stern, M.D., Bach, A., Calsamiglia, S., 1997. Alternative techniques for measuring nutrient digestion in ruminants. J. Anim. Sci. 75, 2256-2276.

Südekum, K.-H., Nibbe, D., Lebzien, P., Steingaß, H., Spiekers, H., 2003. Comparative evaluation of the protein values of soybean and rapeseed meals by *in vivo*, *in situ* and laboratory methods. In: Proc. 11th Int. Rapeseed Congr. "Towards Enhanced Value of Cruciferous Oilseed Crops by Optimal Production and Use of the High Quality Seed Components", Copenhagen, Denmark, pp. 1241-1243.

Tamminga, S., Brandsma, G.G., Dijkstra, J., van Duinkerken, G., van Vuuren, A.M., Blok, M.C., 2007. Protein Evaluation for Ruminants: the DVE/OEB 2007 System, CVB documentation report nr. 53. CVB; Lelystad, The Netherlands.

Thomas, C.E. (Ed.), 2004. Feed into Milk: A new applied feeding system for dairy cows. Nottingham University Press, Nottingham, UK.

Touri, M., Kaustell, K.V., Huhtanen, P., 1998. Comparison of the protein evaluation systems of feeds for dairy cows. Livest. Prod. Sci. 55, 33-46.

Van Duinkerken, G., Blok, M.C., Bannink, A., Cone, J.W., Dijkstra, J., Van Vuuren, A.M., Tamminga, S., 2011. Update of the Dutch protein evaluation system for ruminants: the DVE/OEB2010 system. J. Agric. Sci. 149, 351-367.

Volden, H.E. (Ed.), 2011. NorFor – The Nordic Feed Evaluation System. Wageningen Academic Publishers, Wageningen, The Netherlands.

Wulf, M., Südekum, K.-H., 2005. Effects of chemically treated soybeans and expeller rapeseed meal on in vivo and in situ crude fat and crude protein disappearance from the rumen. Anim. Feed Sci. Technol. 118, 215-227.

CHAPTER 5

Effect of extent and rate of wilting on protein degradability, protein value and amino acid composition of grass silage

B. Edmunds*†, H. Spiekers†, K.-H. Südekum*, H. Nussbaum‡, F, Schwarz, R. Bennett††**

*Institute of Animal Science, University of Bonn, Endenicher Allee 15, 53115 Bonn, Germany † Institute for Animal Nutrition and Feed Management, Bavarian State Research Centre for Agriculture (LfL), Prof-Dürrwaechter-Platz 3, 85586 Poing, Germany ‡ Agricultural Centre for Cattle Production, Grassland Management, Dairy Management, Wildlife and Fisheries Baden-Wuerttemberg, Atzenberger Weg 99, 88326 Aulendorf , Germany**Animal Nutrition Weihenstephan, Technical University of Munich, Weihenstephaner Berg 3, 85350 Freising, Germany ††Adisseo Europe-Africa-Middle East, Antony Parc 2, 10, Place du Général de Gaulle, F-92160 Antony, France

Submitted for publication

Abstract

Despite a high crude protein (CP) concentration, the protein value of grass silage is generally poor due to high degradability and inefficient capture of released N by rumen microbes. Wilting grass prior to ensiling decreases rumen degradability and the present study attempted to describe this effect more specifically by wilting grass to four dry matter (DM) contents (200, 350, 500, 650 g kg^{-1}) at two different rates (fast, slow), creating a total of 6 silages and 2 haylages. There was a quadratic relationship ($P < 0.05$) between ruminally undegraded CP (RUP) and DM, with the increase occurring between DM-500 and DM-650. This effect was accentuated by rapid wilting ($P < 0.05$). Chemical fractionation of CP revealed decreases in non-protein-N and increases in true protein fractions (B2 and B3) between DM-500 and DM-650 for both fast and slow treatments, indicating significant retardation of proteolysis was initiated at a DM above 500 g kg^{-1}. Utilisable CP at the duodenum, a measure of feed protein value, was increased by rapid wilting ($P < 0.05$) but was not affected by DM due to a lower content of metabolisable energy. Wilting treatment influenced the amino acid (AA) composition of the silages, but most of these effects were lost in the rumen. The haylages had a higher total AA content ($P < 0.05$) due to the higher level of RUP. The AA composition after rumen incubation was similar across treatments. Conclusively, fast-wilted silage and haylage was superior in terms of protein value and protein quality. Achieving such results in practice may, however, be challenging.

Keywords: silage, rumen, crude protein degradation, dry matter, utilisable crude protein, crude protein fractions, amino acids

Introduction

Grass silage is one of the most commonly used preserved forages in central and northern Europe. However, dairy cows supplied grass silage containing rations have consistently been shown to be inefficient in N utilisation (Givens *et al*., 2004). Although grass silage provides a large proportion of dietary crude protein (CP), protein supplements continue to improve production responses indicating a lack of efficiency of microbial capture of N from silage. The characteristic effect of ensiling is a decrease in water soluble carbohydrates and an increase in non-protein N (NPN; McDonald *et al*., 1991). This means that fewer carbohydrates are immediately available for microbial use in the rumen whilst there is an abundance of readily available N. In other words, the availability of energy and N to rumen microbes is not in synchrony. This can potentially result in a large N loss in urine and limited N supply to support microbial growth as the cell wall is degraded. Slower degrading protein supplements solve this problem; however, the overall increase in dietary N is associated with increased environmental N emissions and monetary costs to the farmer.

Re-evaluating methods used in making silage to improve efficiency of N capture by rumen microbes may offer a cheap solution to the aforementioned problems. Wilting grass before ensiling has been shown to decrease CP degradability (Merchen and Satter, 1983; Van Vuuren *et al*., 1990; Lebzien and Gädeken 1996; Verbič *et al*., 1999), however, results describing the extent of this influence on degradability have been inconsistent and an interaction with the rate of moisture loss was not examined. Wilting has the potential to be a powerful tool not only in improving efficiency of N utilisation but increasing the amount of ruminally undegraded dietary CP (RUP) reaching the duodenum, providing a valuable source of amino acids (AA) to the animal. As the importance of establishing a protein system based on limiting AA heightens, greater understanding of the composition and amount of AA provided by RUP is required.

This study investigated the effect of wilting to four concentrations of dry matter (DM: 200, 350, 500 and 650 g kg^{-1} fresh matter) at two rates of moisture loss (fast and slow) on CP quality and changes in AA composition of ensiled grass. An intensive examination of N components was performed including analysis of: RUP via the *in situ* technique, CP fractions via the Cornell net carbohydrate and protein system (CNCPS; Sniffen *et al*., 1992), utilisable crude protein at the duodenum (uCP) via the modified Hohenheim gas test (modHGT), and AA composition both prior to and after rumen incubation.

Materials and Methods

Preparation of silages

Eight silages were derived from the same parent material (meadow grass: approximately 0.85 perennial rye grass, 0.08 legumes and 0.07 herbs, second harvest, heading) from the 2008 harvest in Aulendorf, Baden-Württemberg, Germany. The grass was subdivided and either wilted thinly spread on black plastic in the sun (fast; F) or on white plastic in the shade (slow; S) to a DM content of approximately 200, 350, 500 and 650 g kg^{-1}, thus creating six silages and two haylages. Weather conditions during the wilting period were sunny and hot, with maximum temperatures around 30°C over the two days required to achieve all treatment DM targets. When the desired DM was reached the grass was chopped at a 20 mm setting and then ensiled in triplicate, without additives, in 1.75 l glass jars according to the scheme for silage testing in Germany (Bundesarbeitskreis Futterkonservierung, 2006). Ninety days fermentation was allowed in a temperature controlled storage room at 25°C. Wilting times of each treatment can be viewed in Table 1. The treatments will accordingly be referred to as: F-200, S-200, F-350, S-350, F-500, S-500, F-650 and S-650.

General analysis

The silages were pooled, freeze-dried and milled through a 3 mm screen for the *in situ* trial and through a 1 mm screen for all other analyses. Proximate analysis was done according to VDLUFA (2004) and method numbers are given. The DM of the forages and incubation residues was determined by oven-drying of a subsample at 105°C (3.1). Ash and crude lipids (CL) were analysed using methods 8.1 and 5.1.1 respectively. Crude protein was determined by Dumas combustion (4.1.2) for original forage material and *in situ* residues and by Kjeldahl (4.1.1) for CP fractionation analysis using a Vapodest 50s carousel (Gerhardt, Königswinter, Germany) for automated distillation and titration. The same equipment was also used to measure ammonia, by distillation, after incubation in rumen fluid as part of the modHGT. Neutral detergent fibre (NDF: 6.5.1; assayed without heat stable amylase) and acid detergent fibre (ADF: 6.5.2) are expressed inclusive of residual ash. Additionally, ADF without residual ash (ADFom) was determined using NIRS for use as a variable in calculating metabolisable energy (ME: GfE, 2008, see Table 1 for calculation). Silages were not corrected for DM losses associated with drying (Weissbach and Kuhla, 1995) as the correction is based on oven-dried, not freeze-dried material.

In situ procedure

Methods describing the *in situ* trial can be read in detail in Edmunds *et al*. (2012a; Chapter 3). Briefly, the procedure followed basic guidelines of Madsen and Hvelplund (1994) with incubation periods of 2, 4, 8, 16, 24, 48 and 96 hours and using three non-lactating German Holstein cows, fitted with rumen cannula, per feedstuff. Cows received a diet of proportionately and approximately 0.22 soy bean meal and mineral concentrate, 0.53 maize silage and 0.26 grass hay (DM basis) at 07:00 and 16:00 h daily in two equal meals meeting maintenance requirements. The number of bags used as replicates changed with incubation time depending on expected degradability and amount of residue required for subsequent analysis. For each incubation time, bags were inserted directly before the morning feed and were immediately immersed in ice-water upon removal. All bags underwent machine washing in cold water and were subsequently freeze-dried. Incubation residues were pooled per cow and incubation time. Three bags that had not undergone any incubation were also machine washed to calculate the washout fraction. Water soluble material was estimated by mixing duplicate samples in 100 ml, 40°C distilled water and then filtered through No. $595^{1/2}$, diameter 270 mm filter paper (Schleicher and Schuell, Dassel, Germany). The equation of Hvelplund and Weisbjerg (2000) was used to correct CP disappearance for small particle loss at each incubation time point. Correction for microbial attachment (MA: g kg^{-1} residue CP) to undegraded feed particles was carried out using the exponential equation of Krawielitzki *et al*. (2006). The A_{max} parameter of the equation, describing maximum MA at time $t \approx \infty$, was estimated by boiling a subsample of the residue ($t \geq 16$ h) in neutral detergent solution to extract microbes (Mass *et al*., 1999). Time specific degradation of CP was calculated as effective degradability of CP (EDP: g kg^{-1} CP) at an assumed passage rate of 0.04 h^{-1} (K_p4) according to McDonald (1981), but with the assumption that no degradation occurred during the lag phase (Wulf and Südekum, 2005). Finally, RUP was calculated as 1000-EDP.

Protein Fractionation

Division of CP into five fractions (A, B1, B2, B3 and C) based on characteristics of degradability was done according to the CNCPS (Sniffen *et al*., 1992) using standardisations and recommendations of Licitra *et al*. (1996). All fractions, including CP, were analysed in triplicate. See Edmunds *et al*. (2012a; Chapter 3) for a more detailed description.

Modified gas test

Methods describing the modHGT (Steingass *et al*., 2001) can be read in detail in Edmunds *et al*. (2012b; Chapter 4). Briefly, the method followed basic procedures of the original Hohenheim gas test (Menke and Steingass, 1988), the modifications being a 2 g l^{-1} increase in $(NH_4)HCO_3$ and a 2 g l^{-1} decrease in $NaHCO_3$ in the buffer solution and measurement of ammonia at the end of each incubation. Rumen fluid was collected from two to three fistulated sheep receiving a 50:50 grass hay:pelleted compound maintenance ration twice daily. One third of the ration was given at 07:00 and two thirds at 15:30 h. Approximately 200 mg DM sample was incubated for 8 and 48 hours. At the termination of the incubation, the entire contents of the syringe (30 ml) was analysed for ammonia N and uCP was calculated as follows:

$$uCP\ (g\ kg^{-1}\ DM) = ((NH_3N_{blank} + N_{sample} - NH_3N_{sample})/weight\ (mg\ DM)) \times 6.25 \times 1000$$

where NH_3N is in mg 30 ml^{-1}, 'blank' refers to rumen fluid/buffer solution without added substrate, 'sample' is the solution with added sample, N_{sample} is N added to the syringe from the measured amount of feedstuff (mg) and weight is the amount of sample weighed into the syringe and calculated to DM.

Biological between run fluctuations were corrected using a protein standard (provided by the University of Hohenheim), which was analysed with every run. The correction follows the same method as that used for gas production (Menke and Steingass, 1988), with deviations of higher than 10% from the reference mean of the standard requiring repetition of that run. Following correction of uCP, values from the two incubation time points were plotted against a log (ln(time)) scale and the resulting regression equation was used to calculate effective uCP to passage rates of 0.02, 0.04 and 0.06 h^{-1}, which will hereafter be referred to as: uCP2, uCP4, uCP6.

Amino acid analysis

The original material and 16 h *in situ* residues, underwent a complete AA profile analysis, performed in CARAT laboratory, Adisseo, Commentry, France. The AA contents were measured by cation exchange chromatography after acid hydrolysis for 24 h (Directive 98/64/EC, 3/09/99 – Norme NF EN ISO 13903, Antony, France). Analysis of methionine was performed after initial oxidation of samples with performic acid. Phenylalanine was analysed without oxidation.

Correction for added AA coming from microbial colonisation of *in situ* residues was performed using the following procedure. The amount of microbial matter was estimated for each residue as described in the section: '*in situ* procedure'. Next, the AA composition of the microbes had to be estimated. Microbial matter has been shown to be relatively consistent in its AA composition (Storm and Ørskov, 1983; Chamberlain *et al*., 1986) and, although this assumption has been debated (Clark *et al*., 1992), it was taken advantage of in the present study and values published by Storm and Ørskov (1983) were used to correct each sample. As not all N in microbial matter is AA, the calculated extent of microbial contamination was multiplied by a factor of 0.8 (Storm and Ørskov, 1983). The resulting number was then multiplied by the published value for each individual AA and subtracted from the measured concentration of the residue AA as in the following calculation:

$$AA_{correct}\ (g\ kg^{-1}\ DM) = AA_i - (MA \times 0.8 \times (MAA_i/1000) \times (CP/1000))$$

where AA_i is the measured concentration of the ith amino acid from the residue (g kg^{-1} DM), MA is the amount of estimated microbial CP of the residue (g kg^{-1} residue CP) as calculated from the equation of Krawielitzki *et al*. (2006), MAA_i is the concentration of the ith AA in microbial matter (g kg^{-1} AA: Storm and Ørskov, 1983) and CP is the concentration of residue crude protein (g kg^{-1} DM). Summation of individual, corrected AA provided the corrected total AA content.

Statistics

All statistical analyses were performed using SAS version 9.1 (SAS, 2002). Linear and quadratic effects, with DM as the covariable and wilting speed as the fixed effect, were used to determine any significant relationships of treatment on RUP and CP fractions. Due to insufficient material from the S-200 treatment for analysis by CP fractionation and modHGT, statistics analysing results from these procedures include treatments with DM 350 to 650 g only. Treatment effects on uCP and total AA were determined using the proc GLM procedure of SAS using least squares means. Differences were deemed significant at $P < 0.05$.

Results and Discussion

Silage quality

Results of the proximate analysis and exact DM at ensiling are presented in Table 1. Crude protein did not change between treatments ($P > 0.05$), NDF and ADF increased with increasing DM by approximately 70 g and 45 g respectively and ME decreased by approximately 1 MJ kg^{-1} DM in the haylages ($P < 0.05$). Buffering capacity was low, at less

than 100 g lactic acid per kg DM, and sugar level at ensiling was above 80 g kg^{-1} DM, indicating good potential for rapid lowering of the pH (Table 1). Upon opening the silages no visible evidence of moulding or warming was present and the aroma was typical of silage fermented primarily by lactic acid bacteria.

Table 1 Wilting time (h), dry matter (DM; g/kg) at ensiling and proximate variables of silages wilted to various DM content at two rates of moisture loss (F = fast, S = slow). Buffering capacity (g lactic acid/kg DM) and sugar content (g/kg DM) of the unensiled material are also included.

Treatment	Wilting time	DM	CP	NDF	ADF	ME[a]	Buffering capacity	Sugar
F-200	3	194	188	417	244	11.0	53	94
S-200	5	193	189	406	227	11.2	53	96
F-350	7	381	189	434	247	11.2	48	104
S-350	31	373	191	444	254	10.8	51	84
F-500	9	499	186	450	256	10.8	47	114
S-500	33	466	195	436	249	10.8	46	93
F-650	26	692	179	484	275	10.1	46	117
S-650	50	669	191	472	275	10.0	45	118

Rumen undegraded crude protein

Quantitative comparisons of RUP are difficult to make with published data as so many factors affect degradability and its estimation. Such factors include N composition of the plant material itself, variations in methodological techniques and errors associated with the *in situ* analysis including presence or absence of correction for microbial attachment. Therefore analysis of the results in this study is focused on trends rather than numbers, although the RUP results do fall within the normal range for grass silage (150-250 g kg^{-1} CP: Universität Hohenheim – Dokumentationsstelle, 1997) in the fast treatments. The slow wilted silages (DM-200 to DM-500 g kg^{-1}) were slightly lower than 150 g kg^{-1} CP (Figure 1a).

The RUP increased quadratically with increasing DM ($P < 0.0001$). Figure 1a clearly shows that there was no effect of DM on RUP up to and including DM-500. At DM-650, RUP increased ($P < 0.001$) by 60 and 78 g for slow and fast wilted treatments respectively. Wilting speed was also significant, with rapid wilting giving rise to a higher level of RUP ($P <$

0.0001). This is not surprising as longer wilting times lead to more extensive proteolysis. The increase in RUP with DM supports findings from Van Vuuren *et al*. (1990) and Lebzien and Gädeken (1996). However, Van Vuuren *et al*. (1990), who analysed silages with DM contents of 220, 300 and 450 g kg^{-1}, observed RUP increases between all DM levels. Lebzien and Gädeken (1996) also observed a curvilinear increase in RUP with increasing DM in grass silage with the sharp decline in degradability occurring between DM 390 and 600 g kg^{-1}. The threshold DM at which the sharp decline in degradability occurs is not clear and, although the present study suggests this is at DM > 500 g kg^{-1}, it is most probably influenced by multiple factors.

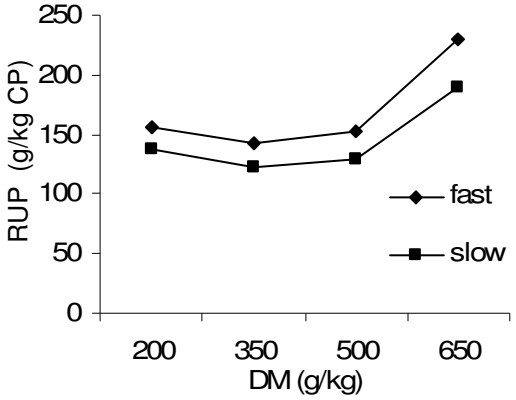
Crude protein fractions

To more clearly understand the effect of wilting on the degradability of protein, CNCPS CP fractionation was performed. Unfortunately insufficient material remained from S-200; however trends between fast and slow were similar between the higher DM treatments for the A, B2 and B3 fractions so a rough idea can be obtained through results of F-200.

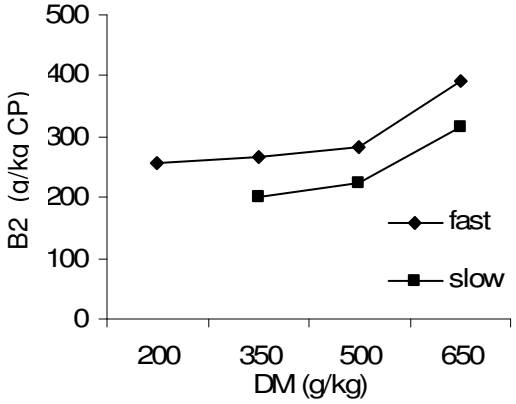
Non-protein nitrogen

The A fraction, which represents NPN, decreased quadratically with increasing DM ($P < 0.0001$). As is characteristic of silages, NPN was high (approximately 600 g kg^{-1} CP) in F-200 and both DM-350 silages. This was also true for the DM-500 treatments. In both haylages NPN was largely reduced ($P < 0.001$), indicating decreased degradation by proteolysis. Additionally, F-650 had a lower proportion of NPN (300 g kg^{-1} CP) than S-650 (450 g kg^{-1} CP) and this trend extended, though not so extremely, to the lower dry matter contents (Figure 1b). Muck *et al*. (1987) demonstrated that the rate of proteolysis of lucerne in-silo decreases linearly with increasing DM concentration. Naturally, a faster rate of moisture loss will induce earlier retardation of proteolysis, which explains the higher level of true protein (TP: CP - NPN) in the fast wilted silages. However, following this principle one would expect a lower concentration of NPN in the S-500 treatment. Muck (1988) observed a similar response and explained it as an interaction between DM and its subsequent effect on the growth of lactic acid bacteria and time taken to lower the pH. One might also expect NPN content to increase with wilting time due to the longer exposure to proteolytic plant enzymes. However, in the present study, the NPN concentration correlated negatively with wilting time but with the presence of a strong interacting effect of DM. For example, although there was a difference of wilting time of 24 h between F-500 and S-500, the NPN was only higher by 66 g

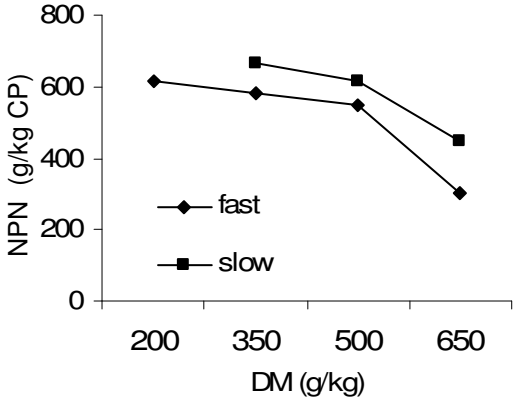
kg^{-1} CP in the slow treatment. Such a small difference between fast and slow treatments probably indicates that most proteolysis occurs during ensiling. It may also be, for the fast wilted treatments, that the decreasing rate of proteolysis with advancing moisture loss was slightly offset by an initial increase in rate caused by higher temperature; a result of wilting in direct sunlight.



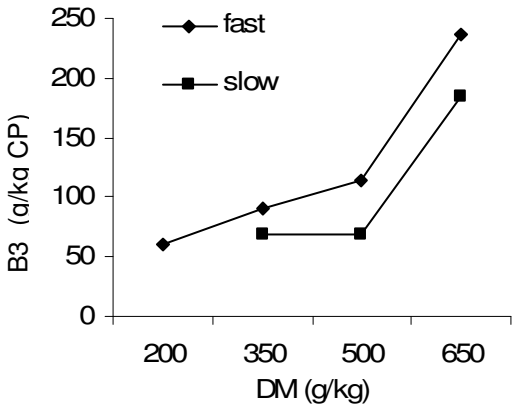
a) Ruminally undegraded dietary protein (RUP) calculated to a passage rate of 0.04 h^{-1}.



c) Intermediately degraded true protein (B2 fraction).



b) Non-protein-N (NPN, A fraction).



d) Slowly degraded true protein (B3 fraction).

Figure 1 Effect of dry matter (DM) at ensiling at two rates of wilting on:

True protein fractions

Treatment had no effect on the B1 (soluble TP) fraction and the concentration was low (mean: 39 g, maximum: 61 g kg^{-1} CP) in all treatments. Fresh, unensiled forage usually contains B1 at above 100 g kg^{-1} CP (Kirchhof *et al*., 2010; Edmunds *et al*., 2012a; Chapter 3) and the low values here indicate its susceptibility to proteolytic attack. Likewise, the B2 fraction suffered losses due to the effects of wilting and ensiling. A study comparing conservation methods and their effects on CP fractions revealed an average B2 fraction of 520 g kg^{-1} CP in fresh forage (n = 12; Edmunds *et al*., 2012a; Chapter 3), whereas the silages in the present study contained an average of 280 g kg^{-1}. The loss of B2 followed a quadratic trend ($P = 0.0001$) with a higher concentration remaining at higher DM and heavier losses resulting from slower wilting ($P < 0.0001$: Figure 1c).

Both linear and quadratic trends ($P < 0.0001$) described the increase in B3 with increasing DM concentration and fast wilting provided higher B3 than slow wilting ($P < 0.0001$: Figure 1d). The sharp rise in B3 between DM-500 and DM-650 resulted in B3 concentrations above that which was found in fresh meadow grass of a similar composition and maturity (Edmunds *et al*., 2012a; Chapter 3). This is in agreement with Nguyen *et al*. (2005) who observed an increase in B3 of 65 g kg^{-1} CP above its fresh counterpart in an orchardgrass silage with a DM content of 600 g kg^{-1}. The reason for the increase in the B3 fraction at low moisture contents is not yet clear. A possible explanation could be a combination of decreased in-silo proteolysis and denaturation of proteins caused by heating: a result of respiration using trapped air. Nguyen *et al*. (2005) suggest sunlight exposure during wilting alters the properties of proteins and forms bonds between proteins and carbohydrates. However, the B3 fraction also increases through conserving forage as hay or through rapid, artificial drying at high temperatures. Artificially dried forage often has limited sunlight exposure; therefore the increase in B3 may simply be a case of decreased solubility caused by precipitation of proteins during drying.

Perhaps the most interesting observation is the relationship between B3 and RUP. Generally, RUP is composed mainly of B3 and C fractions, with some remaining B2. In the present study, regression of B3 + C against RUP revealed a strong linear relationship (n = 7, $R^2 = 0.89$, $P < 0.0001$: Figure 2). There were no differences between the treatments in the C fraction, thus the linearity was entirely due to B3. The one sample that deviated from the trend line was that of the F-200 treatment. Removal of this sample increased the R^2 to 0.99. This

strong trend is more likely to occur in conserved forages due to the depleted B2 fraction and could serve as a rapid and useful estimation of RUP.

The C fraction remained stable (mean = 26 ± 3 g kg^{-1} CP) across all treatments. This is in contrast to the results from Merchen and Satter (1983) who observed significant increases of the C fraction in silage with a DM content of 660 g kg^{-1}. Nguyen *et al*. (2005) also observed a slight increase in the C fraction in silages containing 600 g DM in comparison with moister silages (450 g and 240 g DM), although this increase was very small (98 to 113 g kg^{-1} total N). The stability of the C fraction in this study indicates that heat accumulation during ensiling was not enough to initiate the reaction causing indigestible Maillard products. This is attributable to good compaction and air-tight sealing, which is easy to achieve in experimental glass silos. The results may not be reflected in practice, which is suggested by the results from Merchen and Satter (1983) who used tower silos.

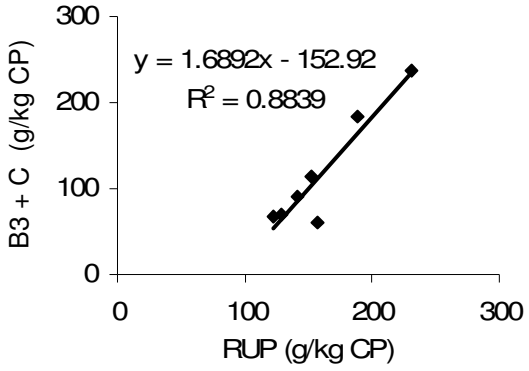


Figure 2 Relationship between RUP and the summation of B3 and C fractions.

Overall the observed trend of decreased protein degradability with increasing DM was in agreement with other studies (Merchen and Satter, 1983; Van Vuuren *et al*., 1990; Lebzien and Gädeken 1996; Verbič *et al*., 1999; Nguyen *et al*., 2005) and this study revealed that rapid wilting and DM above 500 g kg^{-1} reduces degradability and can thus contribute a higher level of RUP at the duodenum. The trial should, however, be repeated under practical conditions as anaerobic conditions are very easy to achieve in small, glass silos.

Utilisable crude protein

Utilisable CP (uCP) is the sum of microbial CP and RUP at the duodenum and it is used in Germany as a measure of protein value (GfE, 2001). Unfortunately, no material remained

from either DM-200 treatments. Initial analysis of uCP revealed some results that went against expectations: F-650 was lower than S-650 at uCP2 (uCP estimated to ruminal passage flow rate of 0.02 h^{-1}). Assessment of nutritional components analysed using the proximate analysis indicated that this was probably due to CP values (Table 1). Although statistically the same, CP appeared to vary more than would be expected for forage coming from the same parent material. Reanalysis reduced the between treatment standard deviation. These discrepancies in CP content made it difficult to obtain a clear picture of the effects of treatment on uCP. Since CP is calculated from total N and total N should not change under such controlled experimental conditions, a singular CP concentration of 186 g kg^{-1} DM (the average CP concentration after reanalysis) was assigned to all samples and uCP was recalculated. The results are presented in Table 2.

Faster wilting yielded significantly higher uCP at all passage rates ($P < 0.05$). The trend existed at all levels of DM (350-650). Such an effect can be explained by reduced proteolysis and a subsequent higher proportion of TP, particularly insoluble TP, entering the rumen. A higher concentration of insoluble TP, namely the B3 fraction, reduces the rate of microbial degradation and increases the amount of RUP entering the duodenum. The higher soluble N content (A + B1) of the slow wilted silages results in a greater increase in the ammonia pool than the fast wilted silages. Utilisation of ammonia by microbes can only occur if there is a suitable carbohydrate source (Nocek and Russell, 1988). Assuming the amount and rate of fermentation of carbohydrates was the same between fast and slow wilted samples *in vitro*, the ammonia pool will remain higher in the slow wilted silages and uCP will consequently be lower.

Utilisable CP increased mildly with increasing DM; however this increase was not significant. From DM-350 to DM-650, uCP4 and uCP6 increased by 6 and 10 g kg^{-1} DM respectively in fast wilted silages. In the slow wilted silages the increase was 8 and 11 g kg^{-1} for uCP4 and uCP6 respectively. The mild increase in uCP, despite a larger proportion of RUP, can be explained by the decrease in ME (approximately 1 MJ kg^{-1} DM) at DM-650 (Table 1). Microbial growth is heavily dependent on rumen available energy and thus the lower RUP in high moisture silages is compensated for by an increased proportion of microbial CP. It must be remembered that the *in vitro* modHGT is a closed system. In *in vivo* situations this compensatory effect may be reduced as a result of early N loss due to the higher proportion of soluble N from lower DM silages. Indeed, an *in vivo* trial by Verbič *et al*. (1999) revealed a larger amount of microbial N reaching the duodenum from sheep fed highly wilted silage

(DM 520 g kg^{-1}) and hay than from unwilted (210 g kg^{-1}) and moderately wilted (430 g kg^{-1}) silage. Additionally, increased intake commonly associated with high DM silages (Dawson *et al*., 1999; Wright *et al*., 2000) will aid in compensating for its lower ME content.

Table 2 Effective utilisable crude protein (uCP; g/kg DM) of silages (n = 4) and haylages (n = 2) wilted to different DM contents at two rates of moisture loss (F = fast, S = slow) and calculated to three assumed rates of passage (0.02, 0.04 and 0.06 h^{-1}) using a fixed CP value of 186 g/kg DM.

	uCP2	uCP4	uCP6
F-350	100	135	155
S-350	96	121	136
F-500	105	138	157
S-500	93	122	139
F-650	101	141	164
S-650	99	130	148
Fast	$102^{a} \pm 2.4$	$138^{a} \pm 3.0$	$159^{a} \pm 4.7$
Slow	$96^{b} \pm 2.9$	$124^{b} \pm 4.9$	$141^{b} \pm 6.2$

Different letters within columns indicate a significant difference ($P < 0.05$).

Amino acid composition

Effects of ensiling

Clear treatment effects on the AA composition of the silages were observed. However, with the knowledge that AA composition of most feeds changes during rumen incubation (Erasmus *et al*., 1994; Van Straalen *et al*., 1997; Von Keyserlingk *et al*., 1998; Gonzalez *et al*., 2001) these ensiling related changes will only be discussed briefly. There was no difference between treatments for total AA content (mean = 131 ± 4 g kg^{-1} DM; P > 0.05), indicating that the plant enzymes were responsible for most of the proteolysis. The mean AA content of CP was 695 ± 31 g kg^{-1}. Figure 3a presents treatment related changes for individual AA. Only DM-350 and DM-650 have been presented for reasons of simplicity. The changes were calculated as percent change from a fresh, unensiled meadow grass sample (Fr-0; second harvest, 2008). The Fr-0 sample was chosen as a representative based on the closenesss of its AA profile to

published values (Degussa Feed Additives, 1996; Misciattelli *et al*., 2002) and was preferred to published values based on geographical location, harvest, and the fact that it was analysed using the same procedure as the silages. Worthy of remark is the higher retention of arginine both with increasing DM and fast wilting. There was an overall decrease in cysteine and methionine. The sharp rise in proline in the slow wilted silages supports findings of Kemble and Macpherson (1954) and is a typical sign of water stress (Boggess and Stewart, 1976). One of the precursors for proline is orthinine (Kemble and Macpherson, 1954) for which arginine is a precursor (Ohshima and McDonald, 1978), which partly explains the large decrease in arginine. Generally, for most AA, concentrations were lower in slow wilted silages. The lack of difference in total AA can be explained by the higher level of proline.

Effects of rumen exposure

Whilst the previously described changes are interesting they may be of little use when trying to establish supply of AA to the duodenum of ruminants. Gonzalez *et al*. (2009), who also reported changes in the AA profile after ensiling, observed that these effects were lost during rumen incubation. Other studies also support this observation (Van Straalen *et al*., 1997; Von Keyserlingk *et al*., 1998) with evidence that the extent of change in the rumen is more specific to the original forage composition. Therefore, it is of interest to know how wilting speed and DM at ensiling affect the AA profile after rumen exposure. The present study analysed *in situ* residues for AA content following 16 h in the rumen.

The averaged concentrations (g kg^{-1} DM) of total and individual AA of the original silage sample (GS-0) and after 16 h rumen exposure (GS-16) are presented in Table 3. Regarding total AA, an average of 31% remained at GS-16. The haylages held a higher total AA content ($P < 0.05$) than all other levels of DM by approximately 44% (61.1 and 58.3 g kg^{-1} DM for F-650 and S-650, respectively), which can be directly related to the higher level of RUP. There were slight, but non-significant, increases in total AA as a result of fast wilting (data not shown). Regarding individual AA, highest losses occurred from alanine and proline, whilst AA showing least amount of degradation were cysteine (67% remaining at 16 h) and methionine (40% remaining at 16 h). The strong hydrophobic nature and disulphur bond of cysteine explains the higher resistance to enzymatic activity (Mahadevan *et al*., 1980).

Changes to the AA profile from GS-0 to GS-16 for treatments DM-350 and DM-650 are further highlighted in Figure 3b. As in Figure 3a, data is presented as percent change which is calculated from data expressed as g/100 g total AA. There appeared to be a mirror effect for many of the changes in AA observed after ensiling (Figure 3a). From the effect of ensiling,

arginine, cysteine and methionine decreased whilst proline increased. The opposite is true after rumen exposure thus all major effects from ensiling seem to be lost in the rumen. Thus, to obtain a clearer picture of the combined effects of ensiling and rumen incubation on the AA composition of RUP, a net change in AA was calculated. Net change is simply the percent change in AA directly calculated from Fr-0 (The original AA profile of an unensiled sample) to GS-16. From this calculation it was immediately apparent that most treatment effects had been lost (data not shown).

The Fr-0 sample had also been incubated in the rumen as part of a larger study using the same materials and methods. The changes from the Fr-0 to Fr-16 (AA composition of unensiled sample after 16 h rumen exposure) and Fr-0 to GS-16 (net changes after ensiling and rumen incubation) are included in Figure 4, where the silages have been presented as a mean for improved clarity. The results are remarkably similar. For all individual AA the percent change from Fr-0 follows the same trend for both Fr-16 and GS-16. Noticeable differences did however occur for arginine, cysteine, histidine, isoleucine, and methionine in that the fresh sample appeared to have a higher level of resistance to degradation than the ensiled samples (except for cysteine, where the silages had a higher retention). Gonzalez *et al*. (2009) also compared AA composition fresh grass (Italian ryegrass) and its silage before and after rumen exposure. Changes in the AA profile after ensiling were reported, however these effects were lost during rumen incubation and the conclusion was that the AA profile of RUP of both forages was similar. The same observation was made in this study. Thus, in estimating supply of RUP-AA to the duodenum, it may be possible to use the AA composition of RUP from a representative sample of similar botanical composition.

Table 3 Average amino acid concentration (g/kg DM) of grass silage and haylage prior to (GS-0; n = 7) and following incubation in the rumen for 16 h (GS-16; n = 7).

	GS-0	±	GS-16	±
Total	130.77	*3.55*	41.09	*12.87*
Alanine	11.07	*0.42*	2.73	*0.88*
Arginine	5.40	*1.69*	1.92	*0.82*
Aspartic acid	13.84	*0.31*	4.62	*1.28*
Cystine	1.26	*0.08*	0.84	*0.08*
Glutamic acid	14.13	*0.87*	4.87	*1.49*
Glycine	7.54	*0.45*	2.66	*0.79*
Histidine	2.79	*0.13*	0.87	*0.32*
Isoleucine	7.00	*0.28*	2.14	*0.63*
Leucine	12.31	*0.80*	4.00	*1.48*
Lysine	7.23	*0.60*	2.55	*0.53*
Methionine	2.34	*0.21*	0.93	*0.31*
Phenylalanine	8.26	*0.26*	2.47	*0.91*
Proline	10.86	*3.26*	2.01	*0.88*
Serine	6.21	*0.25*	2.22	*0.66*
Threonine	6.87	*0.21*	2.44	*0.73*
Tyrosine	4.63	*0.41*	1.13	*0.42*
Valine	9.03	*0.32*	2.70	*0.71*

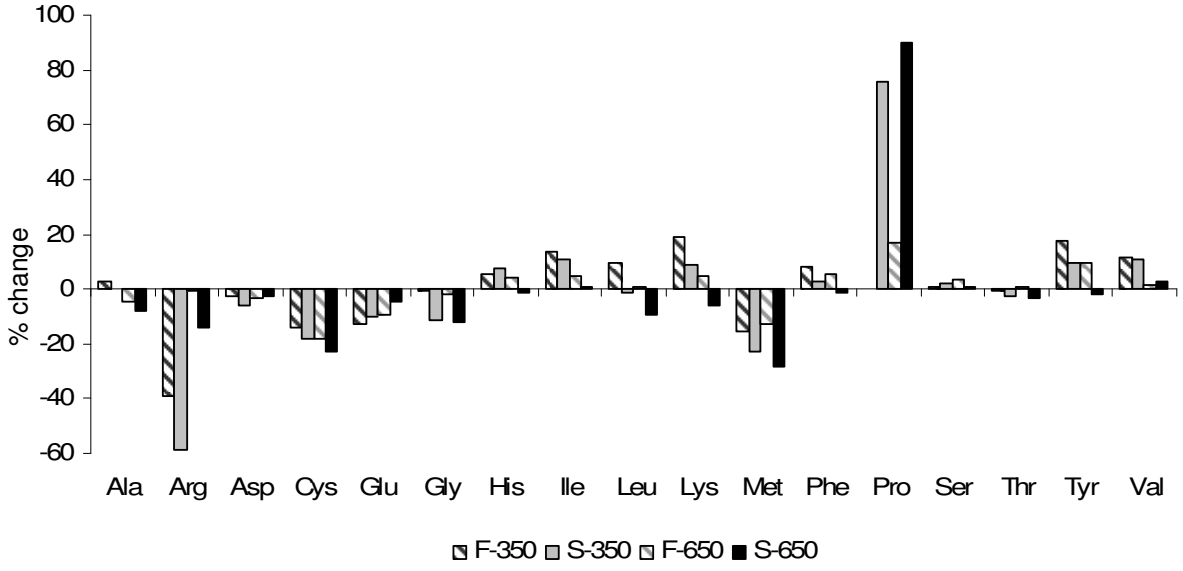


Figure 3 Change (%) in AA composition of silages wilted to various contents of dry matter at two different rates (F = fast, S = slow) as compared to a representative fresh grass sample.

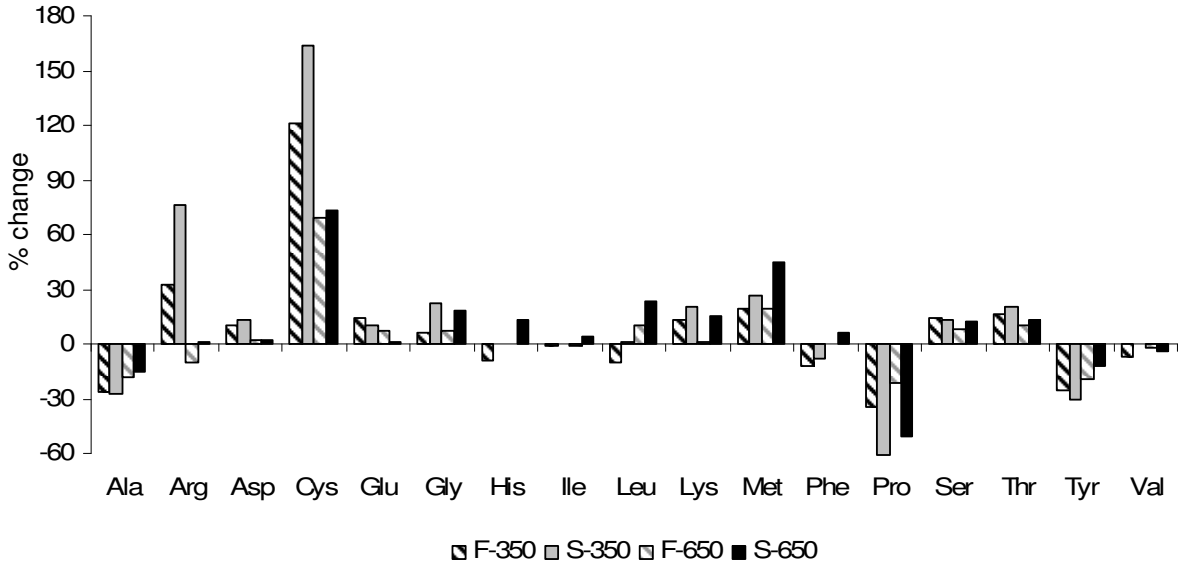


Figure 4 Change (%) in AA composition of silages wilted to various contents of dry matter at two different rates (F = fast, S = slow) from 0 to 16 hours rumen exposure.

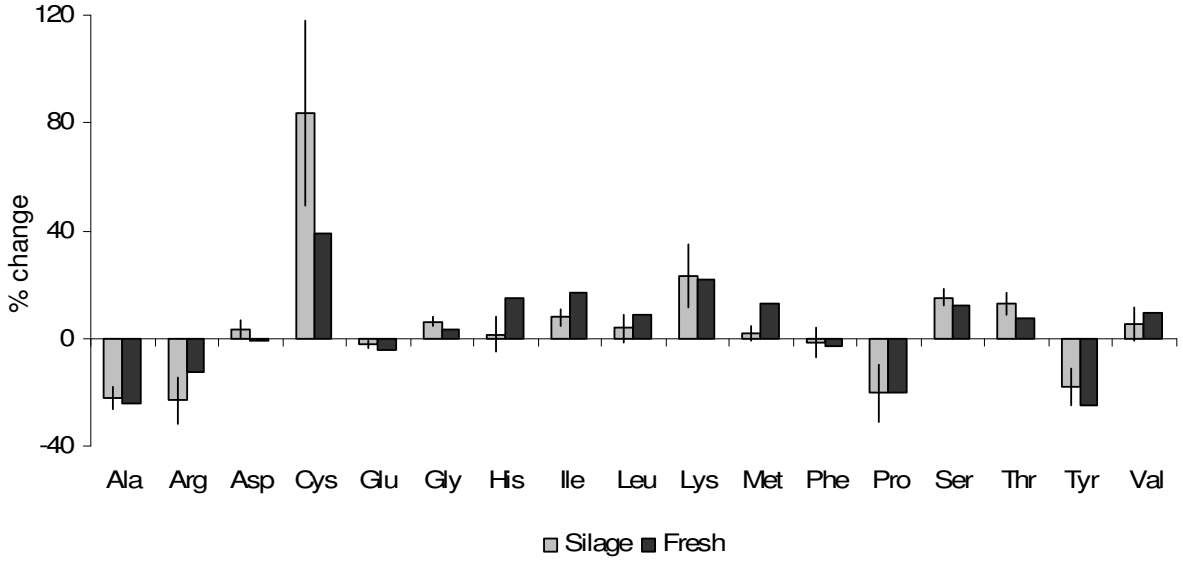


Figure 5 Change (%) in AA after 16 h rumen exposure for fresh grass (Fr-16, n = 1) and silage (GS-16, n = 6). The AA composition of the fresh grass sample at 0 h rumen exposure (Fr-0) was used to calculate net changes in silage i.e. the total effect of ensiling and rumen exposure on the AA profile.

General discussion

Whilst laboratory results from controlled trials suggest high potential for improved protein and AA supply to dairy cows from high DM silages, ensiling of such low moisture grass must be considered in practical conditions. Dry matter of 300 - 400 g kg^{-1} is the usual recommendation (Thaysen, 2004). Ensiling at DM above 400 g kg^{-1} can result in particle loss during transport, low compactibility and warming after opening for use (Nussbaum, 2009). Weather conditions during wilting also pose a problem. It has been well established that rapid wilting favours silage quality as does harvesting at an earlier maturity. The challenge for farmers is finding the ideal time to harvest to optimise these two factors. The use of conditioners may be useful in speeding up the rate of moisture loss during wilting.

Equally as important is the effect of high DM silage on intake and animal performance. Past studies have generally shown an increase in DM intake associated with wilting (Dawson *et al*., 1999), however this and subsequent animal performance is poorly understood. In a review of 85 data sets by Wright *et al*. (2000) positive responses of DM intake, milk energy output and live weight gain were shown to be positively and linearly associated with both the extent and rate of field wilting. More studies are required in this field.

Conclusions

Protein value of grass silage is improved through rapid and extensive wilting. The proportion of RUP and the AA content were significantly higher at a DM of 650 g kg^{-1} and RUP was further improved by a faster rate of moisture loss. Utilisable crude protein at the duodenum was positively influenced by rapid wilting. Increasing DM did not have improve uCP due to a decrease in ME. Finally, although ensiling alters the AA profile, the net effect of ensiling and rumen exposure on the AA profile was not greatly influenced by treatment and the total changes were similar to that of a fresh, unensiled grass of similar botanical composition. This implies that the difference in AA composition between the original material and RUP are due to rumen exposure and not ensiling.

Acknowledgments

This study was financially supported by the Bavarian Ministry for Agriculture, the Institute of Animal Science, University of Bonn and Adisseo, Antony, France. Thanks go out to Dr. M. Schuster and laboratory staff at LfL-Poing for the proximate analysis, E. Devillard, M. Gobert and technical staff at Adisseo for amino acid analysis and staff from LAZBW, Aulendorf, Germany who assisted in the preparation of the silages.

References

ANONYMOUS (1998) Commission directive 98/64/EC of 3 September 1998 establishing community methods of analysis for the determination of amino-acids, crude oils and fats, and olaquindox in feedingstuffs and amending directive 71/393/EEC. *Official Journal of the European Communities*, **L 257**, 14-27.

BOGGESS S. F. and STEWART C. R. (1976) Effect of water stress on proline synthesis from radioactive precursors. *Plant Physiology*, **58**, 398-401.

BUNDESARBEITSKREIS FUTTERKONSERVIERUNG (Ed.) (2006) *Praxishandbuch Futterkonservierung. Silagebereitung, Siliermittel, Dosiergeräte, Silofolien.* 7th ed. Frankfurt/Main, Germany; DLG-Verlag.

CHAMBERLAIN D. G., THOMAS P. C. and QUIG J. (1986) Utilization of silage nitrogen in sheep and cows: Amino acid composition of duodenal digesta and rumen microbes. *Grass and Forage Science*, **41**, 31-38.

CLARK J. H., KLUSMEYER T. H. and CAMERON M. R. (1992) Symposium: Nitrogen metabolism and amino acid nutrition in dairy cattle. *Journal of Dairy Science*, **75**, 2304-2323.

DAWSON L. E. R., FERRIS C. P., STEEN R. W. J., GORDON F. J. and KILPATRICK D. J. (1999) The effect of wilting grass before ensiling on silage intake. *Grass and Forage Science*, **54**, 237-247.

DEGUSSA FEED ADDITIVES (1996) *The amino acid composition of feedstuffs*. Hanau, Germany: Degussa.

EDMUNDS B., SÜDEKUM K.-H., SPIEKERS H. and SCHWARZ F. (2011a) Estimating ruminal crude protein degradation of forages using in situ and in vitro techniques. Submitted for publication.

EDMUNDS B., SÜDEKUM K.-H., SPIEKERS H., SCHUSTER M. and SCHWARZ F. (2011b) Estimating utilisable crude protein from forages using a modified gas test. Submitted for publication.

ERASMUS L. J., BOTHA P. M., CRUYWAGEN C. W. and MEISSNER H. H. (1994) Amino acid profile and intestinal digestibility in dairy cows of rumen-undegraded protein from various feedstuffs. *Journal of Dairy Science*, **77**, 541-551.

GFE (2001) Empfehlungen zur Energie- und Nährstoffversorgung der Milchkühe und Aufzuchrinder. Frankfurt/Main, Germany: DLG-Verlag.

GFE (2008) New equations for predicting metabolisable energy of grass and maize products for ruminants. *Proceedings of the Society for Nutrition Physiology*, **17**, 191-197.

GIVENS D. I. and RULQUIN H. (2004) Utilisation by ruminants of nitrogen compounds in silage-based diets. *Animal Feed Science and Technology*, **114**, 1-18.

GONZÁLEZ J., CENTENO C., LAMRANI F. and RODRÍGUEZ C. A. (2001) In situ rumen determination of amino acids from different feeds corrected for microbial contamination. *Animal Research*, **50**, 253-264.

GONZÁLEZ J., CENTENO C., MORUJO N., FARÍA-MÁRMOL J. and MARTÍNEZ A. (2009) In situ ruminal amino acid degradability of green and ensiled Italian rye-grass according to particle transit model and microbial contamination correction. *Livestock Science*, **123**, 209-214.

HVELPLUND T. and WEISBJERG M. (2000) In situ techniques for the estimation of protein degradability. In: GIVENS D. and OWEN E. (Eds.) *Forage evaluation in ruminant nutrition*. pp. 238-240. Wallingford, UK: CAB International.

KEMBLE A. R. and MACPHERSON H. T. (1954) Liberation of amino acids in perennial rye grass during wilting. *Biochemical Journal*, **58**, 46-49.

KIRCHHOF S., EISNER I., GIERUS M. and SÜDEKUM K.-H. (2010) Variation in the contents of crude protein fractions of different forage legumes during the spring growth. *Grass and Forage Science*, **65**, 376-382.

KRAWIELITZKI K., SCHMIDT T., VOIGT J., KOWALCZYK J. and GABEL M. (2006) Dynamics of microbial contamination of protein during ruminal in situ incubation of feedstuffs. *Journal of Animal and Feed Sciences*, **15**, 313-328.

LEBZIEN P. and GÄDEKEN D. M. (1996) Untersuchungen zum Einfluss des Trockensubstanzgehaltes von Grassilage auf die Abbaubarkeit des Rohproteins im Pansen. *Landbauforschung Völkenrode*, **46**, 169-173.

LICITRA G., HERNANDEZ T. M. and VAN SOEST P. J. (1996) Standardization of procedures for nitrogen fractionation of ruminant feeds. *Animal Feed Science and Technology*, **57**, 347-358.

MADSEN J. and HVELPLUND T. (1994) Prediction of in situ protein degradability in the rumen. Results of a European ringtest. *Livestock Production Science*, **39**, 201-212.

MAHADEVAN S., ERFLE J. D. and SAUER F. D. (1980) Degradation of soluble and insoluble proteins by bacteroides amylophilus protease and by rumen microorganisms. *Journal of Animal Science*, **50**, 723-728.

MASS R. A., LARDY G. P., GRANT R. J. and KLOPFENSTEIN T. J. (1999) In situ neutral detergent insoluble nitrogen as a method for measuring forage protein degradability. *Journal of Animal Science*, **77**, 1565-1571.

MCDONALD I. (1981) A revised model for the estimation of protein degradability in the rumen. *Journal of Agricultural Science*, Cambridge, **96**, 251-252.

MCDONALD P., HENDERSON A. R. and HERON S. J. E. (Ed.) (1991) *The biochemistry of silage*. 2nd ed., Marlow, UK: Chalcombe Publications

MENKE K. H. and STEINGASS H. (1988) Estimation of the energetic feed value from chemical analysis and in vitro gas production using rumen fluid. *Animal Research and Development*, **28**, 7-55.

MERCHEN N. R. and SATTER L. D. (1983) Changes in nitrogenous compounds and sites of

digestion of alfalfa harvested at different moisture contents. *Journal of Dairy Science*, **66**, 789-801.

MISCIATTELLI L., MADSEN J., MOLLER J., THOGERSEN R. and KJELDSEN A. M. (2002) Fodermidlernes indhold af aminosyrer og aminosyrernes andel af aat. *Rapport nr. 9*8. Skejby, Denmark: Landbrugets Radgivningscenter.

MUCK R. E. (1987) Dry matter level effects on alfalfa silage quality. I. Nitrogen transformations. *Transactions of the American Society of Agricultural and Biological Engineers*, **30**, 7-14.

MUCK R. E. (1988) Factors influencing silage quality and their implications for management. *Journal of Dairy Science*, **71**, 2992-3002.

NGUYEN H. V., KAWAI M., TAKAHASHI J. and MATSUOKA S. (2005) Changes in nitrogen fractions and ruminal nitrogen degradability of orchardgrass ensiled at various moisture contents, and the subsequent effects on nitrogen utilization by sheep. *Asian-Australasian Journal of Animal Sciences*, **18**, 1267-1272.

NOCEK J. E. and RUSSELL J. B. (1988) Protein and energy as an integrated system. Relationship of ruminal protein and carbohydrate availability to microbial synthesis and milk production. *Journal of Dairy Science*, **71**, 2070-2107.

NUSSBAUM H. (2009) Futterkonservierung. In: SPIEKERS H., NUSSBAUM H. and POTTHAST V. (eds.) *Erfolgreiche Milchviehfütterung*. pp. 111-203. Frankfurt/Main, Germany: DLG-Verlag.

OHSHIMA M. and MCDONALD P. (1978) A review of the changes occurring in nitrogenous compounds of herbage during ensiling. *Journal of the Science of Food and Agriculture*, **29**, 497-505.

SAS (2002) User's guide: Statistics: SAS Institute, Inc., Cary, NC. USA.

SNIFFEN C. J., O'CONNOR J. D., VAN SOEST P. J., FOX D. G. and RUSSELL J. B. (1992) A net carbohydrate and protein system for evaluating cattle diets: II. Carbohydrate and protein availability. *Journal of Animal Science*, **70**, 3562-3577.

STEINGASS H., NIBBE D., SÜDEKUM K.-H., LEBZIEN P. and SPIEKERS H. (2001) Schätzung des nXP-Gehaltes mit Hilfe des modifizierten Hohenheimer Futterwerttests und dessen Anwendung zur Bewertung von Raps- und Sojaextraktionsschroten. *113th VDLUFA-Kongreß*, Berlin, Kurzfassungen der Vorträge, 114 (Abstr.).

STORM E. and ØRSKOV E. R. (1983) The nutritive value of rumen micro-organisms in ruminants. *British Journal of Nutrition*, **50**, 463-470.

THAYSEN J. (2004) Die Produktion von qualitativ hochwertigen Grassilagen. *Übersichten zur Tierernährung*, **32**, 57-102.

UNIVERSITÄT HOHENHEIM – DOKUMENTATIONSSTELLE (Ed.) (1997) *DLG-Futterwerttabellen für Wiederkäuer*, 7th ed. Frankfurt/Main, Germany: DLG-Verlag.

VAN STRAALEN W. M., ODINGA J. J. and MOSTERT W. (1997) Digestion of feed amino acids in the rumen and small intestines of dairy cows measured with nylon-bag techniques. *British Journal of Nutrition*, **77**, 83-97.

VAN VUUREN A. M., TAMMINGA S. and KETELAAR R. S. (1990) Ruminal availability of nitrogen and carbohydrates from fresh and preserved herbage in dairy cows. *Netherlands Journal of Agricultural Science*, **38**, 499-512.

VDLUFA (2004) Verband Deutscher landwirtschaftlicher Untersuchungs- und Forschungsanstalten - Handbuch der landwirtschaftlichen Versuchs- und Untersuchungsmethodik (VDLUFA-Methodenbuch) Bd. III. *Die chemische Untersuchung von Futtermitteln*. Darmstadt, Germany: VDLUFA-Verlag

VERBIČ J., ØRSKOV E. R., ŽGAJNAR J., CHEN X. B. and ŽNIDARŠIČ-PONGRAC V. (1999) The effect of method of forage preservation on the protein degradability and microbial protein synthesis in the rumen. *Animal Feed Science and Technology*, **82**, 195-212.

VON KEYSERLINGK M. A. G., SHELFORD J. A., PUCHALA R., SWIFT M. L. and FISHER F. J. (1998) In situ disappearance of amino acids from grass silages in the rumen and intestines of cattle. *Journal of Dairy Science*, **81**, 140-149.

WEISSBACH F. and KUHLA S. (1995) Stoffverluste bei der Bestimmung des Trockenmassegehaltes von Silagen und Grünfutter: Entstehende Fehler und Möglichkeiten der Korrektur. *Übersichten zur Tierernährung*, **23**, 189-214.

WRIGHT D. A., GORDON F. J., STEEN R. W. J. and PATTERSON D. C. (2000) Factors influencing the response in intake of silage and animal performance after wilting of grass before ensiling: A review. *Grass and Forage Science*, **55**, 1-13.

WULF M. and SÜDEKUM K.-H. (2005) Effects of chemically treated soybeans and expeller rapeseed meal on in vivo and in situ crude fat and crude protein disappearance from the rumen. *Animal Feed Science and Technology*, **118**, 215-227.

CHAPTER 6 GENERAL CONCLUSIONS

This thesis focussed on improving the accuracy of protein evaluation and understanding of N partitioning from fresh and conserved forages with the distant goal of improving the efficiency of N utilisation in dairy farming. General conclusions drawn are:

1) The method used to correct for microbial attachment to rumen incubated residues shows high potential as a simple and accurate solution to a large source of error in the *in situ* procedure. Further steps are required to validate and standardise this technique.

2) Estimation of RUP by CNCPS protein fractionation is not yet accurate enough to be widely employed. The publicised equations frequently used (Shannak et al., 2000) are based on concentrates and a relatively small data set. There is a strong potential for this method to accurately estimate RUP to various passage rates, provided it is validated against accurate *in situ* or *in vivo* data. To achieve this goal, a much larger data set is required.

3) Estimation of RUP by enzymatic degradation following the standardised protocol of Licitra et al. (1999) shows promise as a quick, reliable and easy to standardise method for routine forage evaluation. Estimation to various passage rates should be possible by adjusting the enzyme concentration and/or incubation time. Further validation is required for a large range of feedstuffs.

4) Estimation of uCP using the modified gas test shows high promise as a rapid, accurate and simple method for evaluation of protein value. The method is sensitive to the biochemical structure and interacting degradation characteristics of individual feedstuffs. Thus it is probably more accurate than the equations currently used in Germany (GfE, 2001). Furthermore, it does not rely on separate analyses of MCP and RUP, and the inherent sources of error associated with their estimation. The procedure is, however, sensitive and care must be taken to follow the protocol exactly and monitor external factors that could influence results e.g. analysis of CP, filling syringes with exactly 30 ml, incubation temperature and time, equipment used for ammonia analysis. Further validation of this method, preferably against *in vivo* data, is required.

5) Wilting grass silage to 65% DM improved the RUP content. This was further improved by rapid wilting. In practice loss of DM during extended wilting, transport and respiration associated with lower compactibility of such dry material may offset the benefits of reduced protein degradability. More importantly, these results may not be replicated in practice due to difficulty in achieving anaerobic stability. Studies under practical conditions are required.

6) The non-protein N content of grass silage decreased with rapid wilting and increasing DM, particularly between 50 and 65% DM. The implications of this are: improved efficiency of N utilisation by the animal and a reduced loss of N through excretion. Studies assessing animal performance are recommended.

7) The RUP and uCP content of grass silage was increased by rapid wilting. Rapid wilting is already a desirable practise in silage making and is largely dependent on weather conditions. What should be considered is whether postponing harvest at the expense of CP content, until favourable weather prevails, is beneficial in terms of final protein value. Increasing DM did not improve uCP due to decreased energy supply. This effect can be easily offset by increasing the energy content of the ration, thus increasing the potential to provide a higher supply of protein to the animal.

8) Amino acid composition was affected by DM content and ensiling. However, most of these affects appeared to be lost after incubation in the rumen. In fact, the amino acid profile of the silage RUP was similar to that of an unensiled sample of similar botanical composition. This observation, if supported by repeated studies, will allow easier and more accurate assessment of the composition of RUP and thus supply of absorbable amino acids from grass silage.

References

GfE, (2001) Empfehlungen zur Energie- und Nährstoffversorgung der Milchkühe und Aufzuchtrinder. DLG-Verlag, Frankfurt/Main, Germany.

Licitra, G., Van Soest, P., Schadt, I., Carpino, S., Sniffen, C., (1999) Influence of the concentration of the protease from Streptomyces griseus relative to ruminal protein degradability. *Animal Feed Science and Technology* 77, 99-113

Shannak, S., Südekum, K.-H., Susenbeth, A., (2000) Estimating ruminal crude protein degradation with *in situ* and chemical fractionation procedures. *Animal Feed Science and Technology* 85, 195-214

Acknowledgments

This study was financially supported by the Bavarian Ministry for Agriculture, the Institute of Animal Science, University of Bonn and Adisseo, France.

Most individuals have already been acknowledged after the paper with which their assistance was associated. In these final words of thanks, I would like to express my gratitude to:

My supervisor Professor Karl-Heinz Südekum for providing me with this opportunity and for his guidance, support, understanding and great sense of humour.

Dr. Hubert Spiekers, Dr. Manfred Schuster, Dr. Karl Rutzmoser, Petra Rauch and staff at the LfL-Bavaria, for their assistance in the first two years of this PhD and for their patience as I came to terms with the basics of the project and struggled (and still struggle!) with the language/s (German/Bavarian).

Dr. Jürgen Hummel and Nadja Wahl (University of Bonn) for assistance in the laboratory and patience as I learned how to conduct myself in the lab in a more economically sustainable manner (sorry about the large number of broken HFT syringes and broken lift!).

My colleagues at the University of Bonn for providing support, empathy, laughs, and some of that great German beer.

Last but most certainly not least I must thank: my friends from Munich, especially Beth, Norberto, Hazel, Breti and Ulli who have encouraged me throughout and cheered me up when times were tough, my family and friends in Australia for the kind words of encouragement and support even though the distance between us was so great, and to my partner, Norman, who gives me so much strength and stability.

CONFERENCES and PRESENTATIONS

2011 Pioneer Silage Conference, Germany, (**Invited speaker**: Optimising protein quality and supply in dairy cow rations)

2011 12th Forum for Applied Research in Cattle and Swine Nutrition, Fulda, Germany (**Poster**: Protein value of silage and hay produced by organic or conventional methods)

2011 65th Symposium for the Society of Nutrition Physiology, Göttingen, Germany (**Oral presentation**: The extent and rate of wilting on protein value and amino acid composition in grass silage)

2010 VDLUFA (German Association for Agricultural Research) Congress, Kiel, Germany (**Oral presentation**: Evaluating the protein value of forages using a modified gas test)

2010 3rd EAAP International Symposium on Energy and Protein Metabolism and Nutrition, Parma, Italy (**Oral presentation**: Evaluating the protein value of forages using a modified gas test)

JOURNAL PUBLICATIONS

Edmunds, B., Südekum, K.-H., Spiekers, H., Schwarz, F. J. (2011) *Estimating ruminal crude protein degradation of forages using in situ and in vitro techniques.* (submitted ANIFEE)

Edmunds, B., Südekum, K.-H., Spiekers, H., Schuster, M, Schwarz, F. J. (2011) *Estimating utilisable crude protein, a precursor for metabolisable protein, from forages using a modified gas test.* (submitted ANIFEE)

Edmunds, B., Spiekers, H., Südekum, K.-H., Nußbaum, H., Schwarz, F., Bennett, R. (2011) *Effect of extent and rate of wilting on crude protein degradability, protein value and amino acid composition of grass silage*. (submitted JGFS)

Edmunds, B., Südekum, K.-H., Bennett, R., Schröder, A., Spiekers, H., Schwarz, F. *The amino acid composition of rumen undegraded dietary crude protein: a comparison between forages.* (Under review)

REPORTS

2010 'Predicting amino acid supply to dairy cows from laboratory methods'
Final report for Adisseo

2010 'Measurement of utilisable crude protein in grass products'
Final report for the Bavarian Ministry for Agriculture