Alternatives for forage evaluation in ruminants

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Avant-propos / Voorwoord

In the first heavy snow of November 1999 John Cone and I went to Clermont-Ferrand to discuss the last details of my PhD project. The result of this final discussion was to continue the cooperation between INRA (Centre de Clermont-Ferrand-Theix, Unité de Recherches sur les Herbivores) and ID TNO Animal Nutrition (later: Animal Sciences Group of Wageningen UR, Nutrition & Food) and to start my PhD study in cooperation with Wageningen Institute of Animal Science. This project started in January 2000. The first 18 months I spent in France and the second part of 30 months in the Netherlands.

My choice for this PhD study was a result of the search for a challenging job with a interesting subject. I was not sure if I would like doing research, but from the beginning I was glad with this move in my career. Moreover, the stay in France for this study was very special and a privilege. The result of a PhD study and writing a thesis is very personal, but impossible without colleagues, family and friends: Thanks!

Jamais je n' oublierais mon arrivée en Auvergne fin Décembre 1999 accompagné par un ami , Roland. Le premier jour (26 Décembre) nous avons été bien accueilli par le propriétaire de l' appartement. Le deuxième jour une grande tempête d' une force rare, et toujours présente dans la tête des Français, était survenue. Le troisième jour, il faisait froid et toute la nature autour de Pardon, mon domicile, était couverte par la neige. Nous avons monté le Puy de Dôme, une marche difficile et mystique avec le givre. Mon séjour en Auvergne était comme ce début: bienvenue, intéressant, varié et en véritable défi dans une belle région. 18 Mois furent trop court et je m'étais bien habitué à cette vie.

Pendant mon séjour, la vie à Theix et Pardon se résumait à beaucoup de travail. J'ai été très bien supervisé par Claude Poncet et Jean Pierre Dulphy, pendant et en dehors des heures de travail. Claude m' a bien introduit dans les sujets de recherche et les discussions avec lui étaient très intéressantes. J'ai réçu de bons avis scientifiques de Jean Pierre, Didier Rémond, Jocelyne Aufère et Raymond Vérité (INRA, Centre Rennes). Les animaux étaient bien soigné et opéré par Didier et Claude.

Ces personnes étaient membres de deux équipes de l' Unité de Recherches dur les herbivores: (URH), VAL (valeur des aliments) et DAN (digestion et absorption des nutriments), dirigées par William Martin Rosset et Michel Doreau respectivement. Les expériences et l'analyse des résultats sont réalisés par des personnes de ces équipes: Marinet Jailler, Eveline Aurousseau, Madeleine Dudilieu, Dominique Graviou, Sophie Gachon, Jaqueline Jamot, Michel Doreau, Pierre Noziere, Béatrice Chauveau, Josiane Chabrot, Michel Jestin, Jean Marie Ballet, Jacques Andrieu, Jean Paul Andrieu, Rogier Bergeault, René Baumont et Camille Demarquilly et les personnes de l'installation expérimentale « Annexes » ; particulièrement Francois

Rosa, Daniel Thomas et Louis L'Hotelier. J' ai bien aimé l'ambiance créé par toutes ces personnes.

L'ambiance et l'humour au travail sont importants. Chaque jour le repas de midi au travail avec Ginette Courtadon, Michel Jestin et Madeleine était important pour moi. Avec eux, avec Eveline, Béatrice, Sophie et avec Cécile Martin, Yvanne Rochette, Ludovic Brossard j'ai beaucoup rigolé.

Je me suis également bien amusé en dehors du travail: sortir avec les autres thésards et chaque samedi faire du vélo avec le club ASPTT. J'ai très bien mangé en France, surtout chez Claude, Jean Pierre, Rogier, Cécile, Ludovic, Eveline, Michel et Brigitte Michalet-Doreau. Je me souviens bien de ces repas, la vendange chez Michel et Brigitte, les week-ends de sport d' ADAS avec Rogier, Ludovic et Michel Fabre et bien sûr le match de foot entre la France et les Pays-Bas regardé avec Pierre et Michel et gagné par les Pays-Bas, bien que la France ait gagné le championnat européen. Le contact avec la famille Vincent en Pardon, le propriétaire de mon appartement, était bon et agréable.

Beaucoup d' humour est lié à la langue et j'ai donc bien aimé parler Hollandais quelques fois, au travail avec Joop et Annette, à Clermont-Ferrand et à l'ASPTT avec Gijs Roos et 100 kilomètres plus loin avec Maria Morselt.

Merci à tous!

De periode in Frankrijk was interessant, gevarieerd, leerzaam, uitdagend met veel plezier in een inspirerende mooie natuur. Het verblijf van 18 maanden was te kort, want je hebt veel tijd nodig om te wennen en in te burgeren naast de drukke werkzaamheden van het onderzoeksproject.

De overgang naar Lelystad is soepel gegaan, niet alleen doordat ik goed opgevangen ben op het werk maar ook door het vinden van een goed huis in een gezellig dorp. In Lelystad kon het AIO-project vlot weer worden opgepakt en kon ik langzaam gaan toewerken naar een proefschrift. Naast het vele schrijven zijn er ook nog een aantal proeven uitgevoerd, welke ik niet zonder de begeleiding van Ton van Gelder en niet zonder het C & E lab had gekund. Dankzij de organisatie van het project door John Cone en de organisatie van personele aangelegenheden door Ad van Vuuren, had het AIO-project een voorspoedig verloop. Belangrijk waren de wetenschappelijke bijdragen van en discussies met Seerp Tamminga en John Cone. Op de afdeling Voeding kon ik ook altijd terecht met inhoudelijke vragen, met name bij André Bannink, Ad van Vuuren en Henk Valk.

Ik was zeer blij met de dagelijkse discussies over alle aspecten van onderzoek en publiceren met AIO-collega John de Leeuw. In de divisie heb ik veel plezier gehad met alle collega's, vooral tijdens de dagelijkse lunch en wandeling. Goede collega's en werksfeer zijn zeer belangrijk, maar minstens zo belangrijk is de stimulans van en het plezier met familie en vrienden. Om niemand te vergeten noem ik verder geen namen: Iedereen bedankt!

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Abstract thesis:

Gosselink, Jules M.J., 2004. Alternatives for forage evaluation in ruminants.

The first objective of this thesis was to validate and to compare *in situ* and *in vitro* techniques with data from *in vivo* experiments. The second objective was to evaluate and to implicate these techniques for future and practical use in feed evaluation. Techniques were compared concerning the ruminal digestion of single forages and concerning the energy values: *in vivo* total tract organic matter digestibility (OMD) and rumen fermentable organic matter (FOM), and protein values: microbial nitrogen synthesised in the rumen (MNS) and rumen escape N (REN). *In vivo* data of 12 forages were investigated using sheep with cannula in the rumen, duodenum and ileum using the markers ^{51}Cr-EDTA, ^{103}Ru-Ph and ^{15}N. Also *in vivo* OMD of 98 forages was measured. The *in situ* nylon bag technique, the pepsin-cellulase technique, the gas production technique (GPT) and the Tilley and Terry technique showed good potency to predict OMD and the *in situ* technique and GPT gave most accurate FOM predictions. MNS was evaluated using *in vivo* data from the literature and showed a significant relationship with the content of crude protein in forages. The method of conservation of forages had an extra effect on MNS. Several determinations of indigestible N and models estimating REN using *in situ* and *in vitro* techniques were compared with *in vivo* REN data. Only ADF insoluble N (ADIN) was related with *in vivo* REN and this relationship improved when fresh and conserved forages were separated. In the general discussion the latter relationships with MNS and REN were implicated in an alternative approach of forage protein evaluation and the alternative techniques were evaluated for practical use in present feed evaluation and future use in mechanistic rumen models.

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Part I

General Introduction

Introduction

1. Objective

Optimising rations for ruminants to increase the farmer income is a challenge since the domestication of ruminants. Nowadays optimal rations should not only result in a high production of milk and meat from healthy ruminants using nutrients from cheap feeds, but also in a reduced waste of nutrients to the environment. The evaluation of rations or feeds can be based on the response of the animal and on the availability of nutrients after absorption from the digestive tract or after digestion in the digestive tract. The current feed evaluation systems are based on the availability of nutrients after digestion of feed, as the measurement of the animal response is expensive and often not possible and the measurement of the absorption of nutrients needs more research.

For the development and the maintenance of these evaluation systems, data from *in vivo* experiments are used. Before 1860, fecal digestibility trials started at the Weende Experimental Station of the University of Goettingen in Germany (Schneider and Flatt, 1975). The measurement of the digestion in the different compartments of the digestive tract using intestinal cannulated ruminants became important about 30 years ago (Tamminga and Chen, 2000). However, to improve and to validate the feed evaluation systems and to evaluate new feeds, *in vivo* data are generally not available.

In vivo experiments have disadvantages as they are laborious and expensive, they have complex methodologies resulting in variable values and they often investigate a ration and not a single feed. Therefore the search for alternative techniques started and was intensified since the increasing concern about animal welfare. Moreover this search was parallel to earlier search for more simple techniques to evaluate feed and also to standardise feed evaluation. In the 19th century laboratory techniques were developed to characterise the composition of feed in chemical terms, as developed by the Weende Experimental Station of the University of Goettingen in Germany (Schneider and Flatt, 1975). The past 40 years *in vitro* techniques and the *in situ* technique using nylon bags were frequently used for research and feed evaluation (Beever and Mould, 2000). However, validations of these alternative techniques and direct comparisons of more than two techniques using the same feed are scarce.

The first objective of this thesis was to validate and to compare alternative techniques using data from *in vivo* experiments. Most frequently used techniques in France and the Netherlands were compared and validated based on feed values from the protein evaluation system for ruminants in France and the Netherlands (Vérité et al., 1987; Tamminga et al., 1994). A part of this, a study of the literature was used to investigate forage values by collecting and interpreting *in vivo* data. The results are compared to the results of alternative techniques. The second objective

was to evaluate and to implicate the alternative techniques for future and practical use in feed evaluation.

The objectives primarily concerned the rumen part of the digestion process. The rumen is the ruminant most specific organ and it has the most complex digestive function to mimic. The objectives were performed using single forages, which were re-evaluated in a large project (INRA-Theix, France). The forages were legumes (lucerne and red clover) and grasses (perennial ryegrass, orchard grass and grass from natural grassland), fresh or conserved as silage, dried forage or hay.

2. Feed values

In a joint project of INRA (Unité de Recherches sur les Herbivores, Theix, France) and ASG (Animal Sciences Group of Wageningen UR, Lelystad, the Netherlands), the feed values from the French and Dutch protein evaluation systems (Vérité et al., 1987; Tamminga et al., 1994) were used to evaluate the alternative techniques. These values are the energy values: total tract organic matter digestibility (OMD) and rumen fermentable organic matter (FOM), and the protein values: microbial nitrogen (N) synthesised in the rumen and rumen escape N. In this thesis N represents protein, as the amount of crude protein is assumed to be 6.25 * N.

OMD is frequently used as a measure of energy supply to ruminants, whereas the FOM part of it delivers energy for microbial N synthesis in the rumen. In the French system FOM is calculated from OMD by subtracting crude fat, rumen escape protein and fermentation products of the ensiling process. In the Dutch system FOM is calculated from OMD by subtracting crude fat, rumen escape protein, rumen undegraded starch and 50 % of the fermentation products are subtracted. In forages starch is negligible, thus the difference between both systems is 50 % of the fermentation products in silages.

Protein supply in ruminants is expressed as protein (n * 6.25) digested from the intestine (PDI in France and DVE in the Netherlands). Important components of this supply are microbial N synthesised in the rumen and rumen escape protein. Microbial N synthesised in the rumen can be calculated from energy by multiplying FOM with the efficiency of microbial N synthesis, in France 23.2 and in the Netherlands 24 gram microbial N per kg FOM. The deficit or the utilisation of rumen degradable N for microbial N synthesis is indicated by the rumen degradable protein balance (Dutch OEB), which is the difference between the availability of rumen effective degradable N and microbial N synthesis calculated from FOM. The rumen effective degradable N is determined as described by Michalet-Doreau et al. (1987), using the measurements from the *in situ* nylon bag technique, which were fitted according to the model of Ørskov and McDonald (1979). From this determination rumen escape N is estimated by multiplying rumen effective undegradable N with 1.11. The coefficient “1.11” is obtained by regression analysis (Vérité et al., 1987).

3. *In vivo* and alternative techniques

3.1. *In vivo* techniques

Generally *in vivo* techniques measuring rumen digestion use sheep or cows surgically fitted with simple cannula in the proximal duodenum to sample digesta leaving the forestomachs. The digesta flow is measured using one marker or two markers. The one marker technique uses mostly Cr_2O_3 or the internal markers, lignin or indigestible acid-detergent fibre. The double marker technique of Faichney (1980) uses mostly the following two markers: one of the liquid associated markers such as Cr-EDTA, ^{51}Cr-EDTA and Co-EDTA and one of the particle associated markers such as Ru-phenanthroline, ^{103}Ru-phenanthroline, Ytterbium or Cr-mordanted neutral detergent fibre. The methodology of the *in vivo* experiment used in this thesis is described in the second part of the general introduction (chapter 1).

The duodenal flows of DM, OM or N measured with the *in vivo* experiment are endpoint measurements of the rumen function. Thus alternative techniques should preferable not only result in endpoint measurements but also mimic the dynamic rumen function. The rumen is a complex digestive compartment to mimic as a result of many dynamic processes concerning the flow of nutrients and the rumen environment (Tamminga and Williams, 1998). The flow of nutrients depends on feed intake, mastication, substrate degradation and storage, microbial growth, passage, absorption and endogenous OM. The rumen environment depends on salivation, buffering, compartmentalisation and stratification.

A result of these effects is that *in vivo* techniques measures apparent digestion of nutrients and most alternative techniques measures true degradation of nutrients. Therefore a mathematical model is needed to transfer the data from alternative techniques into apparent rumen digestion. Another result of those effects is that *in vivo* data can vary a lot and can have high standard errors (Vanzant et al., 1996), although the *in vivo* values used in this thesis showed low variances, as the ratios SD / mean and SEM / mean were lower than 6 %.

A different *in vivo* technique to determine the microbial N synthesis in the rumen is that of Chen and Gomes (1992). This technique measures the urinary excretion of derivatives from the degradation of intestinal absorbed purines originating from microbial nucleic acids. The measurement of the urinary excretion of these purine derivatives proved to be useful in many studies on microbial protein production from forage feeding (Tamminga and Chen, 2000).

3.2. Alternative techniques

In this paragraph the alternative techniques are described and compared with the *in vivo* ruminal environment. The possibilities of these techniques for future use in feed evaluation are discussed in the general discussion.

3.2.1. *In situ* techniques

The *in situ* technique is mimicking the *in vivo* situation more than the *in vitro* techniques, as this technique takes profit from the rumen environment. The *in situ* technique uses nylon bags to incubate feed samples in the rumen of rumen fistulated animals during different periods. The residues of the nutrients from incubated feed in the bags are measured and fitted using the first order model of Ørskov and McDonald (1979). From this model the degradation and the degradation rates of these nutrients in the rumen are calculated:

(1) Disappearance (%) of nutrient at $t = a + b\,(1 - \exp^{-c*t})$

a = soluble fraction of the nutrient (%), estimated from the model or measured by washing or rinsing; b = degradable fraction of the nutrient (%); c = degradation rate per hour; t = incubation time. The model of Ørskov and McDonald (1979) was modified by Robinson et al. (1986) to avoid that a plus b exceeds 100:

(2) Residue (%) of nutrient at $t = u + d\,(\exp^{\{-kd*(t-T0)\}})$

u = undegradable residue of the nutrient (%) after 336 hour of incubation; a = soluble fraction of the nutrient (%), estimated from the model or measured by washing or rinsing; d = 100 - u – a = degradable fraction of the nutrient (%); kd = degradation rate per hour; t = incubation time.

In this thesis the model of Ørskov and McDonald (1979) was used. For the comparison with other techniques an endpoint measurement of rumen effective degradability of a nutrient (Deg6) was calculated with an assumed passage rate (kp) (Michalet-Doreau et al, 1987) using the following model:

(3) $Deg6 = a + [(b * c) / (c + kp)]$

Disadvantages of the *in situ* technique are the reduced animal welfare associated with an inserted cannula, microbial contamination of feed residues in the bags, lack of mastication of the feed samples and the porosity and the rumen location of the nylon bags (Michalet-Doreau and Ould-Bah, 1992). The methodology of different *in situ* experiments differs often in the measurement of the solubility (fraction a), pore size or sample size to bag surface ratio, whereas these factors can have a great effect on the results (Michalet-Doreau and Ould-Bah, 1992). For the calculation of Deg6, the assumptions of a fixed passage rate and that all soluble nutrient is degraded in the rumen are also often criticised. These factors of discussion and the disadvantages make standardisation of the technique necessary, because generally the most important limitations of *in situ* measurements are its low repeatability and reproducibility (Hvelplund and Weisjberg, 2000).

3.2.2. *In vitro* techniques

In vitro techniques can be better standardised than *in vivo* and *in situ* techniques, but they lack more aspects of the *in vivo* rumen environment than the *in situ* technique, no stratification and salivation, buffering in advance and end products are not or partly removed (Tamminga and Williams, 1998). Of all *in vitro* techniques, the *in vitro* techniques using rumen fluid are most close to *in vivo* circumstances, the technique of Tilley and Terry (1963) and the gas production technique (GPT). After incubation of feed in rumen fluid, the technique of Tilley and Terry uses an extra step with incubation in pepsin, but still results in an endpoint measurement.

The GPT is more dynamic as gas is released and the gas production can be measured continuously. In this study a fully automated time related gas production apparatus was used (Cone et al, 1996a). With this apparatus the measurement of gas production during incubation resulted in gas production curves, which can be fitted with a multi-phasic sigmoïdal equation:

(4) $\text{ml gas} = A^1 / (1 + (B^1 / t)^{C1} + \ldots + A^n / (1 + (B^n / t)^{Cn}$

A = maximal gas production in ml; B = time at which half of the maximal gas production (A) is reached in h; C = parameter determining the shape of the curve; t = time in h; n = number of phases in the model. In this thesis the three-phasic model is used as it is considered as the best description of the phases in rumen fermentation, fermentation of the soluble fraction and the non-soluble fraction and the turnover of microbial population (Cone et al., 1997). This model can deliver endpoint measurement of gas production from the different phases as well as kinetic parameters from the model (b and c), which can be used to study fermentation kinetics of different feedstuff.

From the parameters b en c, the fractional rate of substrate digestion (R = /h) at time t can be calculated, if a fixed linear relationship is assumed to exist between substrate fermentation and gas production (Groot et al., 1996):

(5) $R = Ct^{C-1} / (B^C + t^C)$

The time after the start of the incubation at which R is maximal (t_{RM})can also be calculated from B en C (Groot et al., 1996):

(5) $t_{RM} = B * (C – 1)^{1/C}$

The other *in vitro* techniques which were compared in this thesis, all results in endpoint measurements of the degradation of feed components after incubation of feed with enzymes and after chemical characterisation. The enzymatic techniques are the pepsin-cellulase technique for the measurement of DM degradation (Aufrère and Demarquilly, 1989) and the technique using protease for the measurement of

protein degradation (Aufrère and Cartailler, 1988; Cone et al., 1996b). The techniques of chemical characterisation were the determination of neutral detergent fibre (NDF) and acid detergent insoluble N (ADIN) (Goering and Van Soest, 1970; Van Soest et al., 1991). They have one similarity with the rumen function, namely the degradation of feed or feed components.

Fractions of feed components determined with *in vitro* techniques can be implemented in a model to calculate the effective rumen degradation of feed components, using assumptions for degradation and passage rates (Cornell Net Carbohydrate Protein System: Sniffen et al, 1992).

4. Outline of the thesis

In vivo values of the digestion of forages in sheep were the core data of this thesis, as they are the reference values to validate and to compare alternative techniques and to evaluate forages from the literature. Therefore the first chapter (chapter 1a) deals with the performance of the *in vivo* experiment investigating the digestion of six legumes in different digestive compartments: “*In vivo* ruminal and intestinal digestion in sheep fed lucerne or red clover, fresh or conserved by different methods”, and is a part of the general introduction. The methodology of this experiment was also used to investigate six grasses and these *in vivo* values are presented in chapter 1b. This chapter presents also the original data used in chapter 2 for the prediction of the *in vivo* total tract digestibility (OMD). OMD of 98 forages were determined with sheep. From the literature also *in vivo* data from experiments using cannulated sheep or cows and different flow measurements were collected.

The next 4 chapters focus on the alternative techniques for forage evaluation in ruminants and are divided in a part for energy evaluation and a part for protein evaluation. Part II with the alternatives for energy evaluation has two chapters: chapter 2 about the prediction of OMD and is called “Prediction of forage digestibility in ruminants using *in situ* and *in vitro* techniques” and chapter 3 about the prediction of FOM or “Comparison of *in situ* and *in vitro* techniques to predict in vivo fermentable organic matter of forages in ruminants”. These two chapters compare the *in situ* technique, the technique of Tilley and Terry (1963), the gas production technique and the pepsin-cellulase technique using the *in vivo* values from chapter 1.

Part III with the alternatives for protein evaluation has also two chapters about the feed values concerning the synthesis of microbial N in the rumen (chapter 4) and the rumen escape N (chapter 5). The synthesis of microbial N in the rumen was evaluated using *in vivo* data and chemical composition collected from the literature. These data were from experiments investigating a single forage and using cannulated sheep and cows and different digesta flow measurements. This chapter is called: “Estimation of the duodenal flow of microbial nitrogen in ruminants based on the chemical composition of forages: a literature review” .

Chapter 5 deals with the prediction of rumen escape N and is called: “Rumen escape nitrogen from forages in sheep: comparison of data from *in vivo*, *in situ* and *in*

vitro techniques". For this comparison the *in vivo* data of the six legumes and five grasses from chapter 1 were used. Moreover the values of the synthesis of microbial N measured with the technique of Chen and Gomes (1992) were used. Rumen escape N was also determined with the *in situ* technique, the protease technique, ADIN and the model from the Cornell Net Carbohydrate Protein System and they were compared with the rumen escape N estimated from *in vivo* data and from data measured with the technique of Chen and Gomes (1992).

In the general discussion (part IV) the alternative techniques are evaluated for future use and implicated for practical use in forage evaluation. This implication is focussed on protein evaluation of forages and is validated using a database of 77 forages, also containing the values of the synthesis of microbial N measured with the technique of Chen and Gomes (1992). For this implication the approach from the French and Dutch protein evaluation system is used as well as an alternative approach to relate different flows of N fractions entering the duodenum to other more easily determined characteristics of the feed.

Chapter 1a

In vivo ruminal and intestinal digestion in sheep fed lucerne or red clover, fresh or conserved by different methods

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Submitted

In vivo ruminal and intestinal digestion in sheep fed lucerne or red clover, fresh or conserved by different methods

Abstract

Two groups of six mature wethers with cannula in the rumen, duodenum and ileum, were used to study the digestion of lucerne and red clover. Fresh and ensiled forages, lucerne hay and red clover haylage were fed to the sheep. Digesta flows and ruminal outflow rates were measured using ^{51}Cr-EDTA and ^{103}Ru-Ph and as microbial marker ^{15}N was infused. The digestibilities of OM, NDF and ADF in the rumen and total tract were higher for red clover than for lucerne and were higher for fresh red clover compared to conserved red clover. Fresh lucerne had a very low quantity of OM apparently digested in the rumen (OMADR) compared to fresh red clover. Ruminal outflow rates of ^{51}Cr-EDTA and ^{103}Ru-Ph were higher for lucerne than for red clover and decreased in the order of fresh, ensiled and dried forages. The rumen N balance, gram NAN flow at duodenum per kg N intake, was lower for lucerne than for red clover and only red clover haylage had a balance higher than 1000. The yield of microbial N synthesis in the rumen (g N·kg^{-1}of DM intake) was similar between forage species but higher for fresh forages (19.8) compared to the conserved forages (15.4). The efficiency of microbial N synthesis in the rumen (g N·kg^{-1} of OMADR) was similar for red clover forages (average 39.2), but was significantly different between lucerne forages (fresh: 70, silage: 42 and hay: 52). The part of feed N escaping ruminal degradation increased from 110 to 380 with red clover and from 200 to 270 g N·kg^{-1} of N intake with lucerne in the order of fresh, ensiled and dried forages. The amount of N absorbed in the small intestine was similar between the conserved forages (13.2 g N·kg^{-1} DM intake) and higher for the fresh forages.

Keywords : duodenum / nitrogen / organic matter / legume / silage / hay

1. Introduction

Legumes are a protein source in ruminant nutrition. These home-grown feeds make farmers less dependent from the purchase of other protein sources. This is an advantage for the farm economy and ecology, particularly because of restrictions concerning the environment. Moreover, plant proteins become increasingly important since the prohibition of the use of animal protein in livestock nutrition. The capacity of legumes to fix nitrogen (N) from the air results in high protein contents, particularly in lucerne. Among legumes, red clover is promising because of the evidence of a natural protection of protein in red clover, which may result in improved protein

supply to the animal and reduced quantities of N lost in excreta (Wilkins and Jones, 2000).

As a consequence of the high rumen degradability of legume protein, a great part of the nitrogen may be lost by excretion in the urine (Merchen and Bourquin, 1994), unless sufficient fermentable energy is available to capture this N in the rumen in microbial protein. Thus the quantities of nitrogen and energy must be balanced in ruminant rations. However, for composing balanced rations, our knowledge about the quantitative aspects of the availability of legume protein and energy in the different digestive compartments is limited. Not only the amount of experimental results is limited, but also the experiments are heterogeneous in methodology. The absence of sufficient data from experiments with similar methodology complicates the search for reference values for the digestion of forages and explanations of the differences in digestion between forages with forage characteristics, like species, stage of maturity and method of conservation.

Beever et al. (2000) found an effect of the method of conservation on the synthesis of microbial protein in the rumen, which was according to expectations. However, the explanation of the observed differences in rumen protein degradation between methods of conservation is less straightforward (Merchen and Bourquin, 1994). Merchen and Bourquin (1994) created a database of 52 observations out of 28 experiments to characterise protein digestion in forages. But also from this database only some general conclusions were possible: protein is mainly degraded in the rumen, bypass protein is about 26.7 % of protein intake, stage of maturity has only effect in late stages and protein in legumes is more rumen degradable than protein in grasses.

The objective of this study was to obtain reference values for the digestion of lucerne and red clover in ruminants and to compare these legumes using in vivo experiments with sheep. The ruminal and intestinal digestion of organic matter (OM), crude protein (CP) and neutral detergent fibre (NDF) in forages were evaluated. Fresh and conserved forages, as silage and hay, were tested but red clover hay had to be replaced by red clover haylage because of the weather. Haylage is a wilted forage wrapped in bales with plastic, has a dry matter content of about 500 g $\cdot kg^{-1}$ forage and is an alternative for hay when the circumstances during harvesting are wet.

2. Animals, materials and methods

2.1. Forages, animals and experimental design

Two groups of 6 Texel wethers were used to study different forms of lucerne (Medicago sativa) and red clover (Trifolium pratense) in two years, in which for each species the same group of animals was used. The first year, 3 forms of lucerne and the second year 3 forms of red clover were studied in 6 in vivo experiments.

These first cut forages were harvested at the stage between button (about 50 % of the stems have a button) and the beginning of flowering (maximal 10 % of the stems have a flower), which correspond to the ensiling stage. In the week of harvest, both species were studied as fresh forage and conserved as silage with formic acid 80 % (3.5 and 5 litres·ton^{-1} for lucerne and red clover, respectively) and as dried forage. The dried conservation form was sun-dried hay for lucerne and haylage for red clover, which was wilted to 50 % DM content and wrapped in bales with plastic.

At least 2 months before the experiment, the mature wethers were surgically fitted with a ruminal cannula (silicone rubber, 50 mm i.d.) and a T-shaped cannula (silicone rubber, 17 mm i.d.) in the proximal duodenum and the terminal ileum. Sheep were 2 years old and their mean (± SEM) weights during the experiment of each forage were; fresh lucerne: 49.3 (1.38), lucerne silage: 59.7 (1.35), lucerne hay: 55.6 (0.96), fresh red clover: 53.4 (1.69), red clover silage: 58.3 (1.52), red clover haylage: 61.2 (1.33).

The experimental period for fresh forages was in the beginning of June and the periods for conserved forages were in the autumn. Each experimental period lasted 5 weeks. Sheep were allowed to adapt to the forage during the first 2 weeks. The fresh forages were harvested each two days, chopped (5 cm) and stocked at 4°c. The silages were chopped (5 cm) during ensiling and hay or haylage was chopped directly before feeding. The forages were given in 2 equal meals at 12h-intervals (08.00 h and 20.00 h).

The third week was for adaptation of the animal to the metabolic cages. The animals had free access to water and to a mineral block. In the third week the infusion of markers to measure flow and microbial protein were started to establish a plateau. In the fourth week sampling started to measure simultaneously total tract digestibility and intestinal digesta flow. In the last week passage rates of rumen particulate and liquid phases were measured.

During these last 3 weeks, the animals were maintained under continuous lighting in an air-conditioned room (17 – 20 °C) and the feed intake was measured daily. Initially, the feeding level was sub ad libitum, but in the first year the feed intake levels of fresh and ensiled lucerne were unexpectedly high and the intake of lucerne hay was lower. Consequently the intake level of the 3 forms of red clover was fixed at about 1300 g DM ·d^{-1} per sheep.

2.2. Measurements

The daily amount of forage offered was estimated roughly each day by determining the DM content in a microwave and was estimated precisely after the experiment by determining the DM content in a forced air oven on a representative sample taken when the meals were prepared. Refusals were individually weighed and kept frozen until analysis.

In the fourth week of the experimental period the sampling for the total tract digestibility and the intestinal digesta flow measurement were done simultaneously

Table I. Dry matter (DM, $g \cdot kg^{-1}$) and chemical composition ($g \cdot kg^{-1}$ DM) of the six forages.

	Lucerne			Red clover		
	Fresh	Silage	Hay	Fresh	Silage	Haylage
Dry matter	162	220	856	124	174	508
Organic matter	863	901	882	887	903	889
Nitrogen	31.7	29.1	27.4	26.9	25.6	20.5
Neutral detergent fibre	498	438	560	492	478	475
Acid detergent fibre	346	328	379	348	343	352
Lignin	87.0	73.0	85.0	67.5	63.0	55.0
OM – (6.25 * N) – NDF	167	281	151	227	265	286

over 6 days. Daily, faecal excretion was weighed and a representative sample over the week was kept frozen. Daily urine excretion was conserved with sulfuric acid (50 ml H2SO4, 30% v/v) to prevent ammonia losses. It was weighed and diluted with distilled water to a volume of 4 litres and a pooled sample over the week was kept frozen.

Duodenal and ileal digesta flows were estimated using the double marker technique (Faichney, 1980). Solute and particle markers were ^{51}Cr-EDTA (25 µCi $\cdot d^{-1}$ per animal) and ^{103}Ru-phenanthrolin (6 µCi $\cdot d^{-1}$ per animal), respectively. They were continuously infused into the rumen, via separate lines, at a rate of 100 $g \cdot d^{-1}$. The infusion started four days before digesta sampling with a priming dose of 120 ml and stopped in the last week for passage rate measurement.

During 6 days in the fourth week, two samples per day were taken simultaneously from the duodenum and ileum at 6 hr intervals, such that each 1h interval of the 12 h feeding cycle was represented. Immediately after sampling, duodenal (160 ml) and ileal (80 ml) digesta were subsampled under thorough mixing. One fraction (40 ml) was kept as whole digesta. A second fraction (40 ml) was squeezed dry through a nylon gauze (250 µm pore size) resulting in filtrate and particulate subfractions. The remaining fraction from the duodenal digesta (80 ml) was kept for separation of microbes from this digesta. All fractions were pooled per animal and kept frozen until analysis.

The result of microbial separation was a microbial sample, necessary to calculate the fraction of non ammonia nitrogen (NAN) from microbial origin, which is used for the determination of microbial N flow at the duodenum. The microbes in this sample were marked with ^{15}N enriched (> 98%) ammonium sulphate, which was continuously infused (35 mg ^{15}N in 100 ml solution $\cdot d^{-1}$ per animal) into the rumen. This infusion started 2 days before the period of intestinal digesta sampling and continued until the end of this period. For microbial separation, duodenal digesta were thawed and centrifuged (800 g, 10 min at 4°C) to remove feed particles. The supernatant was

spin again (800 g, 10 min at 4°C). The second supernatant was centrifuged at 27000 g for 20 min at 4°C for precipitation of bacteria, which were mainly liquid associated bacteria. The bacterial pellets were freeze dried before analysis.

At the beginning of the fifth week the measurement of the fractional passage rate in the rumen started. At the first day, 3 rumen samples (10.30 h, 13.30 h and 16.30 h) were taken to determine plateau concentrations of the infused flow markers ^{51}Cr and ^{103}Ru. The second day the infusion was stopped before the morning meal and the decline in marker concentrations in time was assessed by sampling rumen contents 5 h after giving the morning meal and then 24 h and 48 h later. To get representative samples from the rumen, they were taken from 4 rumen locations using a concentric tube probe (Faichney et al., 1989), as well as 3 subsamples per sampling time (at sampling time and 30 min earlier and later) were obtained and analysed separately. Marker concentrations among these 3 subsamples were close.

2.3. Analyses and calculations

The contents of DM (104°C, 24 h), OM (550°C, 6 h) and N (Kjeldahl method) were determined on fresh samples of feeds, refusals, faeces and intestinal digesta fractions and on freeze dried microbial pellets. Before analysing, feeds, refusals and faeces were grounded after thawing. Silage and haylage DM was corrected for fermentation products (Dulphy et al., 1975). Ammonia (Weatherburn, 1967) concentration was determined in filtrate and whole digesta from duodenal and ileal samples. Marker (^{51}Cr and ^{103}Ru) concentrations in intestinal samples, faeces, urine, infused solutions and rumen samples were determined simultaneously with a gamma counter (Minaxi γ 5500, Packard). The ^{15}N enrichment in bacterial pellets, duodenal whole digesta and filtrates was determined using an element analyser (Carlo Erba, model NA1500) coupled with a mass spectrometer (Fisons Instruments, model Isochrom). Before the ^{15}N enrichment determination, ammonia was removed from the duodenal samples by adding an equal volume of saturated sodium tetraborate and heating at 95°C for 24 h.

Neutral detergent fibre (NDF) and acid detergent fibre (ADF), were determined on freeze dried samples of feeds, faeces and duodenal whole contents and filtrates (Goering en Van Soest, 1970).

True digesta composition was mathematically reconstituted, using whole contents and filtrates, according to Faichney (1980). Microbial N in duodenal NAN was calculated as the ratio ^{15}N % excess in true digesta / ^{15}N % excess in duodenal microbes. For the calculation of the passage rates in the rumen, the mean marker concentrations of the 3 subsamples per sampling time were taken. The liquid and particle fractional passage rate was the slope of the linear decrease of the logarithm of the marker concentrations of respectively ^{51}Cr and ^{103}Ru after the infusion of these markers was stopped.

Table II. Dry matter (DM), organic matter (OM), neutral detergent fibre (NDF) and acid detergent fibre (ADF) intake and apparently digested in the total tract, rumen, small intestine and large intestine, and ruminal outflow rates of ^{103}Ru–phenantroline (Ru) and ^{51}Cr-EDTA (Cr) of the six forages, and the effect (*P*) of forage species and method of conservation on these parameters.

	Lucerne			Red Clover			SEM	P^{f} (based on fresh and silage)		
	Fresh	Silage	Hay	Fresh	Silage	Haylage		Forage species	Method of conservation	species x method
Intake										
DM ($g \cdot d^{-1}$)	1528 ^{c}	1686 ^{d}	1166 ^{a}	1287 ^{b}	1335 ^{b}	1290 ^{b}				
($g \cdot kg^{-1}$ BW)	31.0 ^{d}	28.3 ^{c}	21.0 ^{a}	24.3 ^{b}	23.0 ^{ab}	21.1 ^{a}				
OM ($g \cdot d^{-1}$)	1329	1519	1028	1141	1206	1148				
NDF ($g \cdot d^{-1}$)	734	737	650	620	681	635				
ADF ($g \cdot d^{-1}$)	505	551	439	525	506	485				
Digestion										
Total tract (TT) ($g \cdot kg^{-1}$ intake)										
OM	592 ^{b}	641 ^{c}	559 ^{a}	725 ^{e}	682 ^{d}	646 ^{c}	6.0	***	NS	***
NDF	548 ^{bc}	522 ^{ab}	504 ^{a}	681 ^{e}	613 ^{d}	541 ^{c}	10.2	***	**	NS
ADF	523 ^{b}	513 ^{b}	463 ^{a}	664 ^{e}	590 ^{d}	526 ^{c}	9.3	***	***	***
Rumen ($g \cdot kg^{-1}$ digestible in TT)										
OM	555 ^{a}	640 ^{bc}	617 ^{b}	714 ^{d}	670 ^{cd}	691 ^{d}	12.5	***	NS	***
NDF	616 ^{a}	682 ^{ab}	760 ^{bc}	835 ^{c}	737 ^{bc}	667 ^{ab}	30.0	***	NS	*
ADF	724 ^{ab}	771 ^{bc}	677 ^{a}	856 ^{d}	733 ^{bc}	817 ^{cd}	32.2	***	NS	***
Rumen ($g \cdot kg^{-1}$ intake)										
OM	329 ^{a}	410 ^{b}	346 ^{a}	518 ^{d}	458 ^{c}	447 ^{c}	15.9	***	NS	***
NDF	333 ^{a}	356 ^{a}	384 ^{a}	568 ^{c}	452 ^{b}	362 ^{a}	17.9	***	NS	**
ADF	378 ^{b}	396 ^{b}	314 ^{a}	568 ^{d}	433 ^{c}	432 ^{c}	19.2	***	***	***
Small intestine ($g \cdot kg^{-1}$ intake)										
OM	204 ^{c}	194 ^{bc}	169 ^{b}	178 ^{bc}	171 ^{b}	137 ^{a}	9.3	**	NS	NS
Large intestine ($g \cdot kg^{-1}$ intake)										
OM	62 ^{c}	38 ^{ab}	45 ^{abc}	29 ^{a}	54 ^{bc}	62 ^{c}	6.8	NS	NS	**
Outflow rates ($\% \cdot h^{-1}$)										
Ru	8.6 ^{d}	6.8 ^{c}	5.0 ^{b}	6.3 ^{c}	4.6 ^{ab}	3.8 ^{a}	0.28	***	***	NS
Cr	12.0 ^{c}	9.6 ^{b}	7.2 ^{a}	8.8 ^{b}	7.2 ^{a}	6.6 ^{a}	0.36	***	***	NS

^{a,b,c,d,e} Means with different superscripts within the same row are significantly different ($P < 0.05$).

^{f} *P* (probability); NS (non significant, $P > 0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Statistical analyses were done with procedures of Genstat® (2000). A multiple pairwise comparison between means was done, based on a normal approximation. A variance analysis was done on data from only fresh and ensiled lucerne and red clover, because the dried conservation form was different for the two species (hay with lucerne and haylage with red clover). The following model was used:

(1) $Y_{ij} = \mu + A_i + B_j + A_i * B_{ij} + \varepsilon_{ijk}$

Here is μ = overall mean, A_i = effect of forage species (i = 1, 2), B_j = effect of method of conservation (j = 1, 2), $A*B_{ij}$ = interaction between forage species and method of conservation, ε_{ijk} = error.

The effect of forage species was confounded with a year effect and the effect of method of conservation was confounded with a period effect. These year and period effects were due to practical circumstances and constraints.

3. Results

3.1. General considerations

The values of the chemical composition of the forages (Table I) were close to the mean values from the feed evaluation tables used in France (INRA, 1988). The decrease in NDF concentration from fresh to ensiled forage was larger with red clover than with lucerne. The protein free non-NDF [OM – (6.25 * N) – NDF] content increased with silage and haylage making.

The differences in DM intake levels (Table II) between the forages have been explained before.

The estimation of the duodenal and ileal flow was reliable. For the entire experiment, the recovery of markers was 1.028 ± 0.038 for ^{103}Ru and 0.960 ± 0.013 for ^{51}Cr. The reconstitution factor R (Faichney, 1980) for lucerne and red clover measurement was respectively 0.260 ± 0.090 and 0.234 ± 0.002 at the duodenal level and 0.243 ± 0.050 and 0.006 ± 0.020 at the ileal level.

3.2. Digestion of OM, NDF and ADF

Differences in digestion in the different compartments of the digestive tract (Table II) were mainly due to forages species and occasionally due to method of conservation. Regardless the method of conservation, total tract digestibility was higher for red clover than for lucerne, and a larger proportion was digested in the rumen. Digestibilities in red clover showed a linear decrease in the order, fresh > silage > haylage. Lucerne silage had higher OM digestibilities in the rumen and total tract than fresh lucerne and lucerne hay.

The contribution of the large intestine to the total tract digestion was of minor importance for red clover as well as for Lucerne.

Table III. Intake of N, duodenal and ileal flow of N, non ammonia N (NAN), microbial N, NANMN (non ammonia non microbial N) or feed escape N and digestion of N or NAN in the small intestines (SI) or total digestive tract of the six forages, and the effect (*P*) of forages species and method of conservation on these parameters.

	Lucerne			Red Clover				P [f] (based on fresh and silage)		
	Fresh	Silage	Hay	Fresh	Silage	Haylage	SEM	Forage species	Method of conservation	species x method
Intake N ($g \cdot d^{-1}$)	50.5	49.0	32.1	34.0	35.4	26.5				
Duodenal flow										
N ($g \cdot d^{-1}$)	46.2	44.0	31.1	32.2	35.0	33.2				
NAN										
$g \cdot d^{-1}$	42.9	40.6	28.6	31.0	32.2	31.3				
$g \cdot kg^{-1}$ N intake	850 [ab]	822 [a]	889 [ab]	911 [b]	910 [b]	1183 [c]	4.8	***	NS	NS
Microbial N										
$g \cdot d^{-1}$	30.4	26.4	18.2	25.2	20.1	19.5				
$g \cdot kg^{-1}$DMI [g]	19.9 [b]	15.7 [a]	15.6 [a]	19.6 [b]	15.1 [a]	15.1 [a]	0.5	NS	***	NS
$g \cdot kg^{-1}$OMADR [g]	70.2 [c]	42.5 [a]	52.0 [b]	42.9 [a]	36.5 [a]	38.1 [a]	2.6	***	***	***
NANMN										
$g \cdot d^{-1}$	12.5	14.2	10.4	5.8	12.1	11.9				
$g \cdot kg^{-1}$ N intake	250 [b]	257 [bc]	322 [cd]	170 [a]	342 [d]	448 [e]	16.2	NS	***	***
Feed escape N [h]										
$g \cdot d^{-1}$	10.2	11.7	8.7	3.9	10.1	10.0				
$g \cdot kg^{-1}$ N intake	204 [b]	236 [bc]	269 [cd]	115 [a]	286 [d]	376 [e]	16.1	NS	***	***
Ileal flow										
NAN ($g \cdot d^{-1}$)	17.9	18.4	12.8	12.8	14.2	15.1				
Digestion in SI										
NAN										
$g \cdot d^{-1}$	25.0	22.2	15.8	18.2	18.0	16.3				
$g \cdot kg^{-1}$ NAN flow at duodenum	582 [cd]	546 [ab]	551 [abc]	587 [d]	559 [bcd]	519 [a]	12.3	NS	**	NS
$g \cdot kg^{-1}$ N intake	494 [ab]	449 [a]	492 [ab]	535 [b]	509 [b]	614 [c]	20.8	**	*	NS
Digestibility total tract										
N ($g \cdot kg^{-1}$ N intake)	705 [d]	709 [d]	679 [c]	699 [d]	654 [b]	560 [a]	6.7	***	**	***

[a,b,c,d,e] Means with different superscripts within the same row are significantly different ($P < 0.05$).

[f] *P* (probability); NS (non significant, $P > 0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

[g] Yield and efficiency of microbial N synthesis in the rumen, resp. g N / kg of DMI (DM intake) and g N / kg of OMADR (OM apparently digested in the rumen).

[h] Feed escape N = NANMN minus endogenous N, which was assumed to be 1.5 gram N per kg of dry matter intake.

3.3. Digestion of nitrogen

Nitrogen intake varied widely among the forages (Table III), due to differences in forage N content (Table I) and differences in DM intake (Table II). Consequently direct comparisons of intestinal flows were not possible. The proportions of N intake recovered as NAN at the duodenum were affected by forage species and were higher for red clover than for lucerne.

The main part of duodenal NAN flow was microbial N: with fresh lucerne and red clover 706 ± 13.2 g $\cdot kg^{-1}$ and 813 ± 15.8 g $\cdot kg^{-1}$ respectively, and with conserved forages 634 ± 6.3 g microbial N $\cdot kg^{-1}$ duodenal NAN flow. This effect of method of conservation was also observed on the yield of microbial N synthesis, expressed as g N $\cdot kg^{-1}$ DM intake, although the yield between the fresh forages was not different (Table III). Forage species affected the efficiency of microbial N synthesis, expressed as g N $\cdot kg^{-1}$ OM apparently digested in the rumen. Lucerne had higher efficiencies, although this effect had an interaction with method of conservation. Fresh lucerne as well as lucerne hay had significantly higher efficiencies than the other forages. A trend of a decreased efficiency with silage making was found

Feed escape N was calculated from the duodenal flow of non ammonia non microbial N (NANMN) assuming a duodenal endogenous N flow of 1.5 g $\cdot kg^{-1}$ DM intake. Feed escape N (proportion of N intake) increased in the order fresh forage < silage < hay or haylage. This increase was more pronounced with red clover than with lucerne. Particularly red clover haylage had very high feed escape N.

NAN digested in the small intestine, as proportion of N intake, (Table III) was higher for red clover than for lucerne. An effect of method of conservation was observed on NAN digestibility in the small intestine. This NAN digestibility tended to be higher with the fresh forages compared to the other forages and was low with red clover haylage. This trend was also found in total tract N digestibility, although lucerne hay had also a low N digestibility (Table III). Total tract N digestibility was also different between the forage species, lucerne had higher N digestibility than red clover. However forage species had an interaction with method of conservation, what is a consequence of the similar values for fresh and ensiled lucerne.

3.4. Digesta outflow rates in the rumen

The outflow rate of ^{103}Ru (Table II), representing the particle fractional outflow rate in the rumen, and the outflow rate of ^{51}Cr (Table II), representing the fractional liquid outflow rate from the rumen, had similar trends. Both rates were affected by forage species and by method of conservation. Lucerne had higher rates than red clover and within each species the rates gradually decreased in the order fresh > silage > hay or haylage, although the difference between red clover silage and haylage was not significant.

Noteworthy were not only the high ruminal outflow rates with fresh lucerne, but also the ratios of liquid phase / particle phase. The mean and SE of these ratios were for all forages 1.50 ± 0.030, for lucerne 1.43 ± 0.020 and for red clover 1.58 ± 0.052.

4. Discussion

4.1. Chemical composition, DM intake and ruminal outflow rates

Comparing lucerne hay with fresh and ensiled lucerne, fed at sub ad lib level, the DM intake of hay was low, probably due to the higher NDF content of lucerne hay. NDF content is well related to DM intake (Van Soest, 1982) and Kawas et al. (1990) attribute this NDF effect on intake to a decreased digestibility and an increased retention time of undigestible residues in the rumen. This higher NDF content as well as the lower CP content of lucerne hay is due to the loss of leaves during harvesting (Merchen and Bourquin, 1994). This loss was also the reason for the low CP content of red clover haylage. The difference in NDF or CP content was smaller between fresh and ensiled red clover than between fresh and ensiled lucerne. Probably the reason was the reduced microbial activity and fermentation in red clover silage caused by the higher dosage of formic acid in red clover silage compared to lucerne silage.

No relation between increased outflow rates and DM intake (g $\cdot kg^{-1}$ BW) was found within all six forages (Table II). Effect of intake level on passage rate in the rumen is limited with forage diets fed above maintenance level (Galyean and Owens, 1991). Lucerne had higher outflow rates than red clover, and thus had an extra effect on these rates, confirming the finding of Vega and Poppi (1997), in which an increasing proportion of lucerne in a diet with lucerne and pangola hay decreases retention time and increases intake markedly. Malbert and Baumont (1989) have suggested a special effect of lucerne on outflow rates, because the increased abomasal outflow with lucerne hay is related to a low viscosity of the contents that are propelled. Kelly and Sinclair (1989) observed a rapid leaf breakdown during eating of fresh lucerne and lucerne hay, which enable the leaves of these forages to pass out of rumen more rapidly than fresh perennial ryegrass and meadow hay, whereas fresh red clover had a intermediate position.

4.2. Digestion of OM and cell wall constituents

Differences in digestion of OM and cell wall constituents (NDF and ADF) were not only due to forage species and method of conservation, but also due to variation in ruminal outflow rates and chemical composition. These variation factors could be interfered by the differences in DM intake between the forages. However the importance of this interference depends on the composition of diets. In mixed diets

ruminal outflow rates increase and ruminal digestion of OM and cell wall constituents (CWC) digestion decrease when DM intake increases (Colucci et al., 1989, 1990; Djouvinov and Todorov, 1994). Other literature showed that effects of intake level on site and extent of digestion of OM and CWC, as well as on passage rate in the rumen, are limited with roughage diets fed at levels above maintenance (Galyean and Owens, 1991; Chilliard et al., 1995). In this study the relation between decreased ruminal digestion of OM and CWC (NDF and ADF) and increased DM intake was not found within the red clover forages and not consistent within the lucerne forages.

The digestion of OM and CWC in the total tract and in the rumen was constantly higher with red clover forages compared with lucerne forages. The hierarchy of these digestibilities in the different presentation forms differed between the species. The literature is poor in in vivo results on digestion of lucerne and red clover forages at the same stage of maturity, what complicates comparisons of values, methods of conservation and species. Two studies found also a higher OM or DM digestibility for red clover compared with lucerne. Lindsay and Hogan (1972) compared dried red clover and lucerne hay at an immature stage of g rowth, but the content of CWC was lower for red clover (390 g $\cdot kg^{-1}$ OM) than for lucerne (540 g $\cdot kg^{-1}$ OM). Dried red clover and lucerne hay had a total tract OM digestibility of respectively 760 and 650 g $\cdot kg^{-1}$ and had a ruminal OM digestibility of respectively 450 and 410 g $\cdot$ kg^{-1}. Kelly and Sinclair (1989) compared fresh red clover and lucerne with similar chemical composition, but the DM digestibilities were respectively 780 and 720 g $\cdot kg^{-1}$. They found a DM digestibility of 540 g $\cdot kg^{-1}$ with lucerne hay, which had almost a two times higher NDF content (579 g $\cdot kg^{-1}$).

Difference in digestibilities between lucerne and red clover was related to the difference in their lignin concentrations. Digestibility declines with increasing lignin concentration (Van Soest, 1982). These differences in digestibility were enhanced by the differences in ruminal outflow rates between lucerne and red clover, because increased ruminal outflow rates caused lower ruminal digestibility. Differences between fresh and conserved red clover might be linked to a change in chemical composition, originating from fermentation of soluble carbohydrates in silage and haylage and from leaf losses in haylage during harvesting. These effects might influence also conserved lucerne forages, but the low digestibility with fresh lucerne was due to the high ruminal outflow rates.

4.3. Digestion of nitrogen

The variation factors, which affect OM digestion, were also important for the N digestion. N content of the legumes was inversely related to the rumen N balance, expressed as g NAN at duodenum g $\cdot kg^{-1}$ N intake. When red clover haylage was excluded the relation ($R^2 = 0.80$) was better than when it was included ($R^2 = 0.46$). Without red clover haylage, NAN at duodenum was equal to N intake when the N content of the legume was 24.6 g $\cdot kg^{-1}$ DM (rumen N balance = 1776 – 13.5 * N

content). Ulyatt et al. (1988) found a higher value (25.5 g $\cdot kg^{-1}$ OM) with a relation (y = 1430 – 16.9 * N content (g $\cdot kg^{-1}$ OM)) using fresh perennial ryegrass and white clover. These values mean net losses of N across the rumen when diets contain more than 24.6 g N $\cdot kg^{-1}$ DM or 25.5 g N $\cdot kg^{-1}$ OM. Red clover haylage had a low N content, what resulted in the utilisation of recycled urea in the rumen N. Species had an effect on rumen N balance because of their N contents.

Method of conservation had an effect when the NAN flow at the duodenum was partitioned in microbial N and in feed escape N. Generally the efficiencies of microbial N synthesis (g N $\cdot kg^{-1}$ OMADR) in this study were higher than the efficiencies found in the literature: from 33 to 58 for fresh forages, from 13 to 28 for silages and a mean between 32 and 33 (Beever et al., 2000). Hays have intermediate efficiencies between fresh and silage (Thomson and Beever, 1980). The differences in efficiencies between experiments are largely due to the differences in methodology. Especially the choice of the microbial marker is important (Siddons et al., 1982). Purine bases are often used as microbial marker. Microbial N production measured by ^{15}N was 18 % higher than estimated from purine bases (Perez et al., 1996). Also important for microbial N measurement is the procedure to isolate and separate free or fixed bacteria from the rumen or duodenum (Yang et al., 1989).

Lucerne and Red clover differ in driving forces for the microbial N synthesis in the rumen. The ruminal OM digestion of red clover was higher and delivered more energy for the microbes to capture degradable N than lucerne. The ruminal OM digestion of lucerne was limited as a result of the high rumen outflow rates. Probably two other mechanisms were also used to deliver energy for the yield of microbial N. At first N was not only used as protein source but also as energy source. Secondly the high outflow rates were favourable for the escape of microbes from the rumen. Low ruminal retention time of microbes decreases the intra-ruminal recycling of microbes by reducing bacterial breakdown and protozoal engulfment (Leng and Nolan, 1984). This mechanism increased the efficiency of the utilisation of energy for microbial N synthesis and might be the reason for the higher efficiencies with lucerne compared to red clover. As a result of the differences in driving forces, the synthesis of microbial N per kg of OM apparently digested in the rumen was similar between the red clover forages and was different between lucerne forages.

In literature, similar trends in the efficiency of microbial N synthesis in the rumen with lucerne with different presentation forms are found. High efficiencies (> 45 g microbial N $\cdot kg^{-1}$ OM apparently digested in the rumen) were found with lucerne hay in steers (Elizalde et al., 1999b) and with fresh lucerne in lambs in combination with high outflow rates of Ru and Cr, respectively 12.0 and 17.7 $\%\cdot h^{-1}$ (Cruickshank et al., 1992). Merchen and Satter (1983) found a higher efficiency for lucerne hay than for lucerne silage.

The average feed escape N from the six forages (248 g N $\cdot kg^{-1}$ N intake) was close to the value found by Merchen and Bourquin (1994), 267 g N $\cdot kg^{-1}$ N intake.

Nevertheless fresh and conserved forages differed significant as well as fresh lucerne and fresh red clover. The high outflow rates with fresh lucerne were the reason for a higher feed escape N (per kg of N intake) compared to fresh red clover. The higher feed escape N with the conserved red clover compared to the conserved lucerne was probably due to the natural protection of protein in red clover as a result of more polyphenol oxidase, an enzyme which reduce proteolysis (Wilkins and Jones, 2000). Consequently a higher proportion of the N intake from red clover was digested in the small intestines than from lucerne N.

The values of N digestibility in the small intestines (g NAN $\cdot kg^{-1}$ NAN flow at duodenum) were close to the mean value of 583, which was found in a database with 67 observations from the literature (not published). Conserved forages had lower values than fresh forages because they had more feed escape N, which contains more non digestible N as a result of drying or loss of leaves during harvesting.

Conserved forages had also a lower yield of absorbed N in the small intestine, 13.2 ± 0.30 g N $\cdot kg^{-1}$ of DM intake, than fresh forages. Fresh lucerne yielded more absorbed N (16.3 ± 0.33 g N $\cdot kg^{-1}$ DMI), than fresh red clover (14.1 ± 0.59 g N $\cdot kg^{-1}$ DMI), as a result of the high N content. But generally the animals didn’t make full profit from the higher N digestibility and content in lucerne compared to red clover: more digestible N from lucerne was lost in the rumen and more digestible N from red clover was protected in the rumen.

5. Conclusion

Lucerne and red clover differ in plant characteristics and in digestion dynamics. Red clover had higher OM digestibility and thus delivered more energy to the animal than lucerne. Compared to red clover, lucerne contains more digestible N and had more efficient synthesis of microbial N in the rumen, but more N from lucerne was lost in the rumen and probably excreted in the urine. The animals utilise N from red clover more efficient than N from lucerne. Conserved forages contain more feed escape N, but had lower N digestibilities in the intestines and the total tract than fresh forages.

The choice between these legumes to compose a ration depends on the requirements. Lucerne is valuable in a diet, when N or high ruminal passage rates are required. Red clover can be chosen when N as well as energy is required. Concerning these specific values of the two species, the fresh forages are most valuable. From the conserved forages the silages are best alternatives, because ensiled lucerne delivers more energy than the other lucerne forages and ensiled red clover had a better protein value than red clover haylage.

Chapter 1b

Databases

Databases

In this chapter two tables with data used to perform the regressions analyses in this thesis are presented. The first table (Table I) presents the *in vivo* values of six samples of perennial ryegrass (*lolium perenne*) and orchard grass (*Dactylis glomerata*), used fresh or conserved as silage and hay. These data were obtained with the same methodology as the six legumes in chapter 1a, except for fresh perennial ryegrass. This ryegrass was not investigated with the double marker technique, but with a technique using only one marker, Ru-phenanthrolin (non radioactive). Comparing these two techniques for the other five grasses and the legumes, the differences in the duodenal flow of OM and non ammonia N were not significant. But the duodenal flow of microbial N was about 5.4% (range = 0.75 to 8 %) lower for the one marker technique. Therefore the *in vivo* data of fresh perennial ryegrass were used in chapter 3 (prediction of fermentable organic matter) but not in chapter 5, describing the prediction of rumen escape N.

Table 2 presents the most important data of database 98 to predict *in vivo* OM digestibility (OMD) using the *in situ* technique (Michalet-Doreau et al., 1987), pepsin-cellulase technique (Aufrère and Demarquilly, 1989), the technique described by Tilley and Terry (1963) and the gas production technique (Cone et al., 1996). The methodology of these measurements are described in chapter 2. Database 98 covers a wide range of forages resulting in a wide range of *in vivo* OMD (Figure 1, the numbers of the samples correspond with the numbers of the samples in table II).

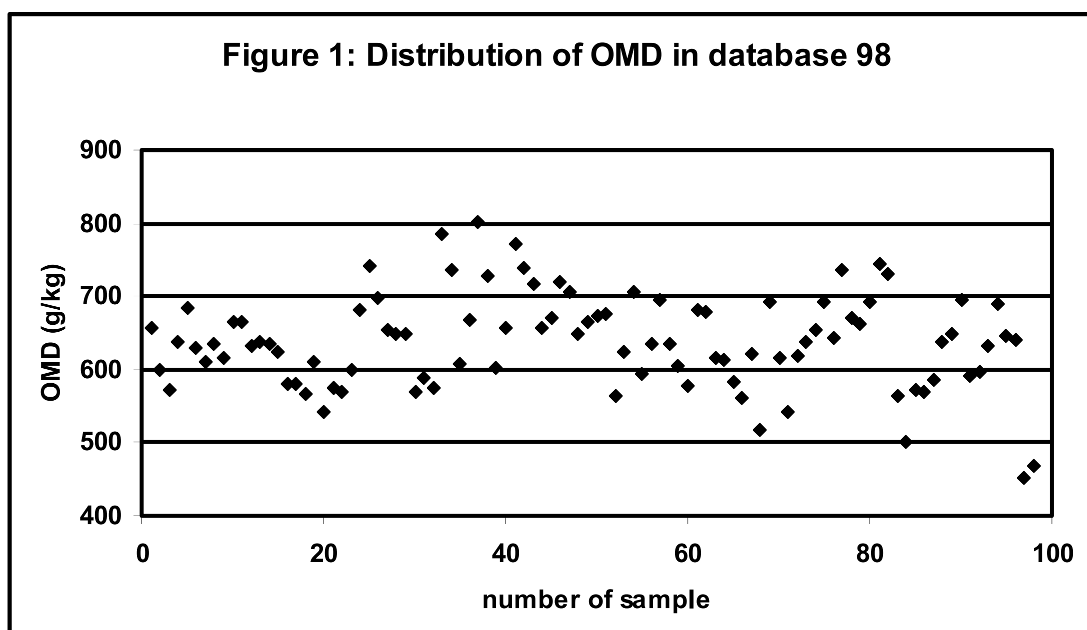

Table I. *In vivo* ruminal and intestinal digestion in sheep fed perennial Ryegrass and Orchard grass, fresh or conserved as silage or hay.

	Perennial ryegrass						Orchard grass					
	Fresh[a]	SD	Silage	SD	Hay	SD	Fresh	SD	Silage	SD	Hay	SD
DM ($g \cdot kg^{-1}$forage)	182		191		873		193		217		852	
CP ($g \cdot kg^{-1}$DM)	91		101		91		116		126		110	
NDF ($g \cdot kg^{-1}$DM)	620		578		632		676		614		697	
DM intake (DMI: $g \cdot d^{-1}$)	1318	0.0	1271	47.6	1300	0.0	1332	0.8	1320	48.0	1161	78.0
OM intake (OMI: $g \cdot d^{-1}$)	1191	0.0	1195	40.9	1162	0.0	1226	0.4	1214	45.2	1078	73.2
OM apparently digested ($g \cdot kg^{-1}$OMI):												
in the total tract	682	15.2	646	14.2	635	5.4	629	8.3	612	16.9	558	22.5
in the rumen	523	36.4	420	36.2	407	37.7	419	26.4	383	27.0	357	18.9
in the small intestine	147	23.4	164	24.2	146	24.5	151	19.4	147	26.3	144	15.5
N intake (NI: $g \cdot d^{-1}$)	191	0.00	20.6	0.66	18.9	0.00	24.6	0.00	26.9	0.78	20.6	1.01
Duodenal flow :												
Non Ammonia N (NAN :$g \cdot kg^{-1}$NI)	1065	128.3	1373	127.9	1429	89.6	1090	60.9	1060	44.9	1179	62.3
Microbial N (MN): $g \cdot kg^{-1}$DMI	133	1.77	15.9	1.35	16.3	1.22	17.5	1.17	16.1	0.58	15.1	1.19
$g \cdot kg^{-1}$OMADR	284	5.36	39.0	6.33	45.3	8.02	45.6	5.64	46.3	4.82	46.1	5.60
NANMN[b]: $g \cdot kg^{-1}$NI	136	15.5	390	60.8	311	16.1	145	19.1	270	30.9	326	32.3
NAN digested in the small intestine												
$g \cdot kg^{-1}$ duodenal NAN flow	591	31.9	621	21.9	613	15.0	610	29.6	614	22.7	600	18.1
$g \cdot kg^{-1}$ NI	628	84.4	855	105.2	877	59.1	665	53.6	652	44.4	709	54.8
N digested in total tract ($g \cdot kg^{-1}$NI)	588	24.4	557	11.9	547	12.8	633	9.3	651	18.8	582	16.6
Outflow rates ($\% \cdot h^{-1}$): ^{51}Cr-EDTA	-		8.35	1.003	7.17	0.319	8.85	1.081	8.76	1.012	7.30	0.788
^{103}Ru-phenanthroline	-		5.27	0.589	5.43	0.510	6.34	0.920	6.17	0.380	5.29	0.643

[a] These values were measured as described in chapter 1, but with one flow marker (non radioactive Ru-Phenanthroline) instead of two flow markers.
[b] NANMN = Non Ammonia Non Microbial N

Table II. DM content (g/kg forage) and content of CP and NDF (g/kg DM), DM intake (DMI: g/kg$^{0.75}$ per day), OM degradation measured *in vivo* (OMD: g/kg), the soluble (a: g/kg) and degradable (b: g/kg) DM fractions and degradation rates (c: /h) measured with the *in situ* technique, DM degradation measured with the pepsin-cellulase technique (pep-cel: g/kg), OM degradation measured with the Tilley and Terry technique (TT, measured values: g/kg) and parameters from the gas production technique (A from the 1st and 2nd phase, B and C from the 2nd phase) of the 98 forages (Dulphy et al., 2003a+b) used in this thesis.

Forage	Method of conservation	DM	CP	NDF	DMI[b]	OMD	*In situ* technique			pep-cel	TT[c]	Gas production technique			
							a	b	c			A1	A2	B2	C2
1. Lucerne	fresh	171	227	408	79.1	657	421	411	0.104	697	681	57.9	125	7.66	2.46
2. Lucerne[a]	fresh	162	198	489	72.7	600	320	410	0.114	631	615	43.6	117	8.31	2.48
3. Lucerne	fresh	159	172	515	64.6	572	364	378	0.118	610	588	45.2	111	8.26	2.37
4. Lucerne	fresh	164	199	459	75.7	638	360	394	0.125	681	633	48.3	118	7.49	2.49
5. Lucerne	fresh	107	182	468	81.0	684	331	510	0.098	665		25.7	111	9.60	2.66
6. Lucerne	fresh	191	165	484	81.0	629	319	467	0.095	613	591	19.7	119	9.29	2.66
7. Lucerne	fresh	280	129	625	82.5	610	326	416	0.088	643	628	52.7	117	7.34	2.41
8. Lucerne	silage	206	168	438	77.3	635	440	334	0.080	624		38.0	119	7.34	2.49
9. Lucerne[a]	silage	212	163	420	76.0	617	380	378	0.077	640	680	42.8	125	7.75	2.44
10. Lucerne	silage	202	183	372	86.2	666	432	381	0.097	700		39.9	134	7.11	2.68
11. Lucerne	silage	212	177	377	94.3	666	408	404	1.101	711		48.5	126	7.07	2.56
12. Lucerne	silage	213	151	448	76.5	632	401	375	0.069	662	635	56.5	124	7.04	2.58
13. Lucerne	silage	216	143	476	77.5	638	380	404	0.059	661	648	59.2	131	7.06	2.63
14. Lucerne	haylage	397	130	509	65.0	634	369	427	0.051	608	660	53.3	127	7.72	2.38
15. Lucerne	haylage	610	141	509	65.1	625	315	441	0.061	615	646	55.0	128	7.66	2.37
16. Lucerne	haylage	700	108	552	83.8	579	247	448	0.058	563	608	53.4	118	7.80	2.27
17. Lucerne	haylage	610	123		81.6	579									

Continued on next page

Table II : continued (1).

Forage	Method of conservation	DM	CP	NDF	DMI[b]	OMD	*In situ* technique			pep-cel	TT[c]	Gas production technique			
							a	b	c			A1	A2	B2	C2
18. Lucerne	hay	881	94	534	85.2	567	244	443	0.079	548	614	25.2	127	11.02	2.69
19. Lucerne[a]	hay	861	165	528	2.5	610	212	491	0.074	605	606	36.7	113	8.50	2.32
20. Lucerne	hay	885	139	554	72.3	541	217	486	0.083	560		46.2	113	8.07	2.29
21. Lucerne	hay	858	176	512	75.3	574	201	496	0.090	625		43.4	114	7.76	2.54
22. Lucerne	dried	913	176	492		570	302	404	0.109	627		54.4	101	6.79	2.39
23. Lucerne	dried	911	189	473		599	321	431	0.121	661		59.7	104	6.72	2.40
24. Lucerne	dried	916	239	374		681	302	545	0.110	732		65.5	110	6.34	2.46
25. Red clover	fresh	87	212	449	70.9	741	410	472	0.168	725		51.2	151	7.89	2.89
26. Red clover[a]	fresh	127	169	492	70.6	699	485	352	0.106	696	700	65.1	135	7.43	2.71
27. Red clover	fresh	169	159	496	73.8	655	451	351	0.141	703		64.1	130	6.82	2.81
28. Red clover[a]	silage	171	157	478	68.4	650	352	457	0.101	649	648	50.0	121	7.68	2.69
29. Red clover[a]	haylage	524	123	475	62.7	650	344	463	0.059	668	703	55.3	136	7.82	2.63
30. Red clover	haylage	469	133	50	51.9	569	325	442	0.074	620		45.6	122	8.15	2.53
31. Red clover	haylage	649	98	523	55.1	589	276	453	0.074	632		49.1	130	8.18	2.50
32. Red clover	hay	850	108	534	65.1	574	294	428	0.090	607		54.1	130	8.17	2.39
33. Ryegrass	fresh	112	171	491	65.4	786	418	515	0.088	765		65.4	163	8.41	2.58
34. Ryegrass	fresh	128	129	551	58.6	736	355	527	0.072	673		53.5	151	9.16	2.57
35. Ryegrass	fresh	178	87	633	47.2	608	318	449	0.049	511		40.7	122	9.88	2.55
36. Ryegrass	fresh	181	92	612	62.4	668	327	509	0.054	538		51.2	133	9.64	2.47
37. Ryegrass	fresh	135	194	512	67.0	803	355	592	0.096	762		61.3	153	8.91	2.62
38. Ryegrass	fresh	165	112	559	51.7	729	365	532	0.070	660		65.5	155	8.86	2.41

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Table II: continued (2).

Forage	Method of conservation	DM	CP	NDF	DMI[b]	OMD	*In situ* technique			pep-cel	TT[c]	Gas production technique			
							a	b	c			A1	A2	B2	C2
39. Ryegrass	fresh	202	83	649	49.1	601	307	470	0.050	465		37.2	119	10.93	2.45
40. Ryegrass[a]	fresh	182	86	620	59.3	657	271	550	0.054	587	700	61.9	148	9.38	2.52
41. Ryegrass	fresh	200	100	513	77.6	771	354	572	0.072	723	762	70.6	159	8.54	2.75
42. Ryegrass	silage	157	128	488	68.7	739	467	462	0.055	700		42.4	143	10.81	2.62
43. Ryegrass	silage	166	110	502	68.9	718	412	506	0.057	680		45.4	148	9.60	2.80
44. Ryegrass	silage	205	87	542	60.9	656	359	521	0.036	578		43.5	130	10.22	2.53
45. Ryegrass	silage	200	87	561	59.6	671	385	499	0.037	586		39.6	121	10.34	2.59
46. Ryegrass	silage	189	121	514	62.4	719	429	503	0.056	695		48.2	157	9.77	2.86
47. Ryegrass	silage	194	119	507	62.2	705	382	535	0.057	691		52.4	161	9.94	2.78
48. Ryegrass[a]	silage	191	100	578	56.5	648	324	497	0.051	583	684	63.0	153	8.52	2.65
49. Ryegrass	haylag	433	96	558	62.3	664	325	524	0.042	604	700	53.4	133	8.80	2.67
50. Ryegrass	haylag	564	79	592	60.8	672	269	644	0.028	574	713	58.8	155	8.74	2.57
51. Ryegrass	hay	839	98	588	57.4	675	302	554	0.048	579		55.5	144	10.01	2.57
52. Ryegrass	hay	869	70	652	49.9	563	212	582	0.036	502		44.4	135	9.99	2.61
53. Ryegrass	hay	860	81	647	54.9	625	57	864	0.039	526		45.8	143	9.98	2.67
54. Ryegrass	hay	846	97	573	55.4	706	300	587	0.050	612		63.4	151	9.60	2.77
55. Ryegrass	hay	875	67	663	43.5	593	198	585	0.040	469		46.7	126	11.12	2.44
56. Ryegrass[a]	hay	873	84	628	67.3	635	248	550	0.053	546	675	63.4	149	9.34	2.49
57. Orchard grass	fresh	196	155	581	62.7	695	338	568	0.080	655	746	59.9	150	8.35	2.68
58. Orchard grass[a]	fresh	193	117	676	55.6	635	224	584	0.051	516	675	47.4	135	9.71	2.66
59. Orchard grass	fresh	212	89	697	51.5	604	212	521	0.045	450	577	36.3	110	10.12	2.59

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Table II: continued (3).

Forage	Method of conservation	DM	CP	NDF	DMI[b]	OMD	*In situ* technique			pep-cel	TT[c]	Gas production technique			
							a	b	c			A1	A2	B2	C2
60. Orchard grass	fresh	325	127	628	70.5	578	201	651	0.051	548	632	54.8	128	9.06	2.52
61. Orchard grass	silage	225	113	587	60.2	682	302	554	0.038	561	677	46.1	138	9.37	2.88
62. Orchard grass[a]	silage	217	121	614	55.5	680	268	593	0.037	556	660	44.2	141	9.50	2.81
63. Orchard grass	silage	295	133	536	59.2	616	345	515	0.049	582	643	45.1	127	8.84	2.78
64. Orchard grass	silage	287	127	545	65.2	613	337	525	0.052	592	635	45.0	130	8.81	2.79
65. Orchard grass[a]	hay	852	110	697	60.5	584	164	665	0.035	462	633	34.6	121	10.54	2.51
66. Orchard grass	hay	875	80	686	54.8	560	161	572	0.040	441	580	35.2	116	9.73	2.44
67. Orchard grass	hay	846	138	560	58.9	621	248	612	0.058	531	672	34.4	114	9.53	2.55
68. Orchard grass	hay	882	97	702	50.0	518	123	692	0.029	424					
69. Natural grass[d]	fresh	197	179	602	66.1	692	317	545	0.051	598	721	47.9	144	10.04	2.60
70. Natural grass[d]	fresh	221	125	652	55.5	615	264	541	0.049	495	644	43.0	124	10.50	2.48
71. Natural grass[d]	fresh	196	108	652	45.0	542	285	496	0.048	434	597	43.9	107	10.72	2.24
72. Natural grass[d]	silage	230	116	575	52.7	618	329	511	0.038	525		43.3	119	11.24	2.53
73. Natural grass[d]	silage	230	115	559	53.7	639	334	517	0.037	557		48.9	134	11.37	2.45
74. Natural grass[d]	silage	248	137	577	58.7	655				565					
75. Natural grass[d]	silage	333	191	530		691				721		58.4	160	8.50	2.88
76. Natural grass[d]	silage	342	131	590		642				602		51.6	138	9.37	2.67
77. Natural grass[d]	silage	294	196	484		737				701		45.8	139	8.81	2.90
78. Natural grass[d]	haylage	301	156	540	53.6	670				569					
79. Natural grass[d]	haylage	617	157	644	55.2	661				535					
80. Natural grass[d]	haylage	530	196	605		691				695		52.5	161	8.68	2.74

Continued on next page

Table II: continued (4).

Forage	Method of conservation	DM	CP	NDF	DMI[b]	OMD	*In situ* technique			pep-cel	TT[c]	Gas production technique			
							a	b	c			A1	A2	B2	C2
81. Natural grass[d]	haylage	347	189	499		743				678		59.4	152	8.40	2.55
82. Natural grass[d]	haylage	437	203	481		730				730		54.4	162	8.55	2.86
83. Natural grass[d]	hay	859	119	679	55.7	564	150	652	0.039	467		34.6	110	11.38	2.60
84. Natural grass[d]	hay	877	86	741	45.6	502	95	624	0.035	386		31.9	95	11.95	2.47
85. Natural grass[d]	hay	881	110	651	56.5	571						42.3	103	10.57	2.30
86. Natural grass[d]	hay	880	109	676	56.5	569									
87. Natural grass[d]	hay	880	80	710	56.5	585									
88. Natural grass[d]	hay	857	117	643		637				566		53.2	137	9.38	2.55
89. Natural grass[d]	hay	840	174	624		649				642		54.2	148	9.28	2.65
90. Natural grass[d]	hay	837	200	617		695				654		50.2	149	8.97	2.73
91. Natural grass[d]	hay	860	58	596	63.1	590				564		49.5	133	10.34	2.41
92. Natural grass[d]	hay	881	103	630	62.2	597				555		52.0	139	9.89	2.48
93. Natural grass[d]	hay	875	110	577	68.0	631				572		20.0	154	11.68	2.71
94. Natural grass[d]	hay	839	196	587		690				631		56.2	152	9.56	2.70
95. Natural grass[d]	hay	877	113	584	72.0	646				610		67.3	156	8.63	2.39
96. Natural grass[d]	hay	875	168	698	85.8	641				633		36.5	156	10.44	2.94
97. Barley	straw	900	33	866	37.0	453	27	763	0.024	315		15.6	95.8	13.45	3.23
98. Rice	straw	900	35	782	42.7	467	44	761	0.021	282		17.7	89.0	12.52	3.23

[a] These 12 forages belongs to database 12.
[b] Correction for seasonal influence on DMI, as described by Dulphy et al (1999)
[c] Not corrected for *in vivo* standards.
[d] Grass from natural grassland.

Part II

Alternatives for energy evaluation of forages in ruminants

Chapter 2

Prediction of forage digestibility in ruminants using *in situ* and *in vitro* techniques

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Abstract

Two experiments were done to determine the *in vivo* digestibility of organic matter (OMD) of forages in sheep. The first experiment was done with 12 forages (database 12) consisting of fresh and conserved forms of lucerne, red clover, orchard grass and perennial ryegrass, fed restricted to sheep. The second experiment was done with 98 forages fed ad libitum to sheep, divided in a database with 37 forages (database 37) with similar qualities as database 12 and a database with 61 forages (database 61). OM and DM digestibility of the forages from these databases was also determined with the *in situ* nylon bag technique, the pepsin-cellulase technique, the technique of Tilley and Terry and the gas production technique. Database 37 was used to find relationships between OMD and the alternative techniques and between OMD and chemical composition. Databases 12 and 61 were used to validate the observed relationships. The databases were also used to find out if there was an effect of DM intake on the relationships. The OMD predictions by the alternative techniques improved and the effect of DM intake disappeared upon the inclusion of a chemical parameter. The prediction by the *in situ* technique plus crude protein content showed highest accuracy in the validations, although the four alternative techniques showed similar potency in predicting OMD. The choice of the technique for prediction also depends on other factors, such as animal welfare, price, time, experience and additional information on feed degradation.

Keywords: nylon bag / gas production / Tilley and Terry / pepsin / cellulase

Abbreviations: ADF = acid detergent fibre; BW = body weight; CP = crude protein; DMI = dry matter intake; MSPE = mean square prediction error; PError = prediction error; NDF = neutral detergent fibre; OMD = *in vivo* organic matter digestibility; RSE = residual standard error.

1. Introduction

In vivo organic matter digestibility (OMD) is defined as the proportion of feed OM apparently digested in the total digestive tract. OMD is a measure of energy available for ruminants and is used in protein evaluation systems (Vérité et al., 1987;

Tamminga et al., 1994) to calculate rumen fermentable OM, which in its turn is used to estimate microbial protein synthesis in the rumen.

In vivo experiments to determine OMD values of forages are expensive and laborious, impair animal welfare and are not suited for routine analysis. Therefore alternative *in vitro* techniques to predict OMD were developed in the past 40 years, such as the *in vitro* technique of Tilley and Terry (1963), the pepsin-cellulase technique (McLeod and Minson, 1978; Aufrère and Michalet-Doreau, 1988) and the gas production technique (Menke and Steingass, 1988). These techniques showed a good correlation with OMD and are used for feed evaluation in the Netherlands, France and Germany, respectively. Also data obtained by the *in situ* nylon bag technique were related with OMD (Demarquilly and Chenost, 1969; Fonseca et al., 1998). The *in situ* and *in vitro* techniques showed good relationships (Givens et al., 1989; Blümmel and Ørskov, 1993; Cone et al., 1999).

Although these commonly used techniques have a good potential to predict OMD (Khazaal et al., 1993; Chenost et al., 2001), their relationships with OMD have not been well validated. However, it is difficult to evaluate the consistency of the relationships of these techniques with OMD or with each other, as long as techniques do not have standard procedures.

The first objective of this study was to investigate the most commonly used alternative techniques in France and the Netherlands in their capability to predict OMD of forages in ruminants. A second objective was to identify the technique that predicted OMD most accurately by comparing and validating these alternative techniques.

Three databases were used to compose and to validate equations for estimating OMD from the pepsin-cellulase technique, the *in situ* nylon bag technique, the gas production technique and the technique as described by Tilley and Terry (1963). These databases were based on quality of the forages. OMD predictions were found using good quality forages, which are important in ruminant production in Western-Europe, and were also tested for low quality forages. OMD of the forages from two of these databases were measured with sheep fed ad libitum, as done in practical circumstances. As a consequence of this, DMI effect is also tested, because alternative techniques mimic only the OM digestion.

2. Materials and Methods

2.1. Forages

Two experiments were performed at INRA in France to determine OMD. In the first, OMD of 12 forages (database 12) was determined. The forages consisted of fresh form, silage and hay of lucerne (*Medicago sativa*), red clover (*Trifolium pratense*), orchard grass (*Dactylis glomerata*) and perennial ryegrass (*Lolium perenne*). Red clover haylage, a wilted forage, baled and wrapped in plastic, with a dry matter

content of about 500 g kg^{-1}, was used instead of red clover hay because of wet harvest conditions.

In the second experiment OMD of these 12 forages together with 86 other forages was determined. This database of 98 forages consisted of a great variety of forages and forage qualities. To obtain more variation in the chemical composition, forages were harvested at different stages of maturity, namely at the vegetative stage, the bud stage and the start of flowering stage. Almost all forages were first cut except one legume and 5 grasses, which were from a second cut.

From this database all alternative techniques were investigated with 37 forages (database 37), as these forages had similar good quality as the forages from database 12 (Table I). These forages are important in the ruminant production in Western Europe. Therefore database 37 was used to find relationships between OMD and alternative techniques and database 12 was used to validate these prediction equations. The rest of the 98 forages was collected in database 61, although the alternative techniques were investigated with only a part of this database (Table II). Database 61 contain also low quality forages and was used to find out if the observed prediction equations are also valid for these forages.

Database 37 contained 15 legumes (12 Lucerne and 3 red clover forages) and 22 grasses (11 orchard grasses, 5 perennial ryegrasses and 6 forages from natural grassland). From these 37 forages 19 were fresh forages (7 legumes and 12 grasses) and 18 were conserved forages (7 silages, 5 hays and 6 haylages with a DM content of 350 or 550 g DM kg^{-1} forage).

Database 61 varied more in forage type and chemical composition than database 37 (Table1). Database 61 consisted of 17 legumes, 12 lucerne and 5 red clover forages, and 44 grasses, being 1 orchard grass, 22 perennial ryegrasses and 21 samples from natural grassland. Database 61 consisted of 7 fresh forages (3 legumes and 4 grasses) and of 54 conserved forages (14 legumes and 40 grasses). Of the conserved forages 17 were silage, 26 were hay, 8 were haylage and 3 were dehydrated lucerne.

2.2. Digestibilty and intake measurement

In both experiments OMD and dry matter intake (DMI) were measured (Table I and 2) in the course of a period of 5 years using Texel wether sheep, weighing between 50 and 60 kg and between 1 and 2 years old. For 3 weeks these animals were housed in metabolic cages with free access to water and a mineral block. The measuring technique differed in the two experiments because of different objectives.

In the first experiment the sheep were surgically fitted with cannula in the rumen, duodenum and ileum, with the aim of investigating the digestion of the 12 forages in the different compartments of the digestive tract. The animals were allowed to adapt to the cages (continuous lighting and 17 – 20 °C) during one week and to the forages during 3 weeks. In the fourth week sampling for the OMD measurement started. The

forages were chopped and given in 2 equal meals at 12h-intervals (08.00 h and 20.00 h). The animals were offered a restricted amount of feed (90 % of *ad libitum*). In the third and fourth week DM intake was recorded daily. Refusals were individually weighed and kept frozen until analysis. For 7 days daily faecal excretion was recorded, collected and pooled in one representative sample, which was kept frozen.

In the second experiment the animals were also fed only one forage *ad libitum*, as the objective was the recording of the voluntary intake. Voluntary intake was measured according to the method of Dulphy et al (1999a), which includes a correction for seasonal effects on intake capacity, because fresh forages were given in spring and conserved forages in autumn and winter. OMD measurement was done according to Demarquilly and Jarrige (1964). Each experimental period lasted 3 weeks. During the first two weeks the animals were adapted to the diet and given two meals per day *ad libitum* (10 % refusals) at 08.00 h and 16.00 h. The third week was used to record voluntary intake and to determine OMD. For OMD measurement, daily faecal excretion was recorded, collected and pooled for one representative sample for 7 days, which was kept frozen.

The contents of DM (80°C, 48 h), ash (550°C, 6 h) and N (Kjeldahl method) were determined on fresh and ground samples of feeds, refusals and faeces. The DM of silage and haylage was corrected for fermentation products (Dulphy et al., 1975). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were determined on the samples dried at 60 °C using the method described by Goering and Van Soest (1970) and were expressed with residual ash. NDF was determined without the use of sodium sulphite and alpha amylase.

Table I. Mean, range and SEM (g kg^{-1} DM) of the chemical composition.

	Database 12			Database 37			Database 61			
	Mean	Range	SEM	Mean	Range	SEM	Mean	Range	SEM	n
CP	133	84 – 198	10.6	133	79 – 227	5.9	133	33 – 239	6.0	61
NDF	558	420 – 697	25.8	557	408 – 697	12.9	571	372 – 866	12.4	60
ADF	325	235 – 426	14.2	341	235 – 426	6.3	333	254 – 485	6.0	60
DMI	645	562 – 821	24.8	712	510 – 934	20.6	628	403 – 918	14.1	58
DMI-cor[a]				663	450 – 852	16.2	616	370 – 943	17.7	48

Abbreviations: CP = crude protein; NDF = neutral detergent fibre; ADF = acid detergent fibre; DMI = dry matter intake (g ·kg$^{-0.75}$ BW).

[a] Correction for seasonal influences on DMI, as described in Dulphy et al (1999a).

2.3. *In situ and in vitro measurement of digestibility*

All samples (Table II) were investigated with the *in situ* nylon bag technique (Michalet-Doreau et al., 1987) and the *in vitro* techniques, enzymatic technique using pepsin and cellulase (Aufrère and Demarquilly, 1989) and gas production technique (Cone et al., 1996). The *in vitro* technique according to Tilley and Terry (1963) was used to determine the digestibility of the forages of only databases 12 and 37 (Table II).

2.3.1. *In situ nylon bag technique*

The method of sample preparation to measure DM degradability with the *in situ* nylon bag method was developed by Dulphy et al. (1999b). Samples of fresh forages and silages were lacerated and put into nylon bags, quickly frozen in liquid nitrogen and conserved at -20 °C, until use. Hays were simply ground to a mesh size of 4 mm and put into the nylon bags.

Rumen incubation procedures were according to Michalet-Doreau et al. (1987). Six samples were incubated in the rumen per incubation time (0, 2, 4, 8, 16, 24, 48 or 72 hours of incubation). Three rumen fistulated cows, fed on a ration of hay (70 % of DMI: DM content of 850 g $\cdot kg^{-1}$ forage, CP content of 106 g $\cdot kg^{-1}$ DM and NDF content of 652 g $\cdot kg^{-1}$ DM) and concentrate (30% of DMI: CP content of 140 g $\cdot kg^{-1}$ DM and NDF content of 290 g $\cdot kg^{-1}$ DM), were used (2 samples per cow). After incubation DM content of the residues in the nylon bags was determined.

The data were fitted with the exponential model of Ørskov and McDonald (1979). The parameters from this model were used to calculate effective degradable DM (Michalet-Doreau et al., 1987), using rumen passage rates (kp) of 0.03 h^{-1}, which is similar to the passage rate of DM in the total tract (W. Pellikaan, personal communication), 0.045 h^{-1} ,used in the Dutch protein evaluation system (Tamminga et al., 1994) and 0.06 h^{-1} ,used in the French protein evaluation system (Vérité et al., 1987) and with kp equal to ruminal degradation rate of DM (kd), since kp as a function of kd (kp = 0.41 * kd) improved the prediction of fermentable OM, as calculated in the Dutch protein evaluation system (Van Vuuren, 1993).

Also undegraded DM, calculated as 100 minus the soluble and degradable fractions, was used in the analyses as well as DM residue in the nylon bags after 72 hours of incubation was used. The DM residue after 72 hours was used, as it had better results in predicting OMD than the other incubation times.

2.3.2. *Pepsin-cellulase technique*

The pepsin-cellulase technique used was the technique developed by Aufrère (1982) and modified by Aufrère and Demarquilly (1989). The DM digestibility of each forage was determined 3 times.

Table II. Mean, range and SEM (g kg^{-1}) of forage digestibility measured with different techniques.

Technique [a]	Database 12			Database 37			Database 61			
	Mean	Range	SEM	Mean	Range	SEM	Mean	Range	SEM	n
OMD	634	558 – 725	14.3	632	542 – 771	7.5	641	453 – 803	9.4	61
Effective degradable DM										
kp = 3	628	520 – 759	18.2	632	486 – 759	10.5	639	357 – 810	18.1	39
kp = 4.5	582	453 – 732	21.2	586	428 – 732	11.5	591	280 – 782	19.6	39
kp = 6	548	407 – 709	23.1	552	388 – 709	12.2	556	241 – 757	20.5	39
kp = kd	549	457 – 661	14.8	555	447 – 661	8.4	564	407 – 698	12.8	39
DM residue after 72 h	308	257 –392	14.1	304	134 – 421	11.9	266	79 – 405	14.7	39
Undegradable DM	202	139 – 297	13.2	197	74 – 313	9.9	174	53 – 303	11.6	39
Pepsin-cellulase	595	462 – 696	19.4	590	434 – 723	12.0	603	282 – 765	13.7	57
Tilley and Terry										
measured	665	606 – 703	9.6	654	577 – 762	7.5				
corrected	651	570 – 701	12.5	630	515 – 788	11.0				
Gas production technique										
Degradable OM	652	536 – 755	17.1	648	536 – 802	11.6	664	446 – 847	12.6	54
Gas production 20h	184	149 – 216	6.7	178	139 – 230	3.7	182	101 – 229	3.8	54
B2	8.0	6.5 – 10.0	0.28	8.8	7.0 – 11.0	0.18	9.3	6.3 – 13.4	0.21	54
C2	2.6	2.3 – 2.9	0.05	2.6	2.2 – 2.9	0.03	2.6	2.3 – 3.2	0.03	54

[a] OMD = OM digestibility in the total tract; *in situ* technique: effective degradable DM (calculated using different passage rates (kp)), undegradable DM and DM residue after 72 h of incubation; DM digestibility from with the pepsin-cellulase technique; OM digestibility from the Tilley and Terry technique with measured and corrected values; gas production technique: degradable OM after 72 h of incubation, gas production after 20 h of incubation, B2 and C2.

2.3.3. In vitro technique according to Tilley and Terry

A generally used technique to determine OM digestibility is the two-stage *in vitro* technique using rumen fluid and pepsin, developed by Tilley and Terry (1963). In this study measured values as well as values corrected with *in vivo* values according to the modification of Van der Meer (1986) were used to predict OMD. The determination of these measured and corrected values was done in duplicate. The rumen fluid was sampled from fistulated sheep fed 800 gram of perennial hay (DM content of 860 g $\cdot kg^{-1}$ forage, CP content of 148 g $\cdot kg^{-1}$ DM and NDF content of 562 g $\cdot kg^{-1}$ DM) and 200 gram of concentrate low in starch (CP content of 187 g $\cdot kg^{-1}$ DM and NDF content of 316 g $\cdot kg^{-1}$ DM).

2.3.4. The gas production technique

The forages of the 3 databases were incubated in four runs in the gas production technique, as described by Cone et al. (1996). Rumen fluid was sampled from the same animals fed the same ration as the rumen fluid used in the technique of Tilley and Terry. Gas production profiles were described with a three-phasic model (Cone et al., 1996; Groot et al., 1996), describing the gas production caused by fermentation of the soluble components (phase 1), the non-soluble but fermentable components (phase 2) and microbial turnover (phase 3). Each sub-curve (phase) was described by 3 parameters, A is the maximum gas production (ml /g OM), B is the time (h) needed to reach 50 % of A and C is a parameter determining the shape of the curve (without dimension).

The gas production (ml /g OM) after 20 hours of incubation, estimated as A from the first phase plus A from the second phase, was provided in the tables as it had the best fit. The parameters B and C from the second phase (B2 and C2) were also provided in the tables, as they improved the precision of the OMD prediction.

After 72 hours of incubation the OM degradation (as g $\cdot kg^{-1}$ of OM incubated) was also determined by filtering the residue over a P1 glass crucible.

2.5. Statistics

Database 37 was used to find the best equations to predict OMD with alternative techniques. These equations were validated with databases 12 and 61. Database 61 was also used to evaluate the effect of DM intake (g $kg^{-0.75}$ BW) on the OMD predictions found with database 37. These regressions, validations and statistical analyses were applied using Genstat (2002). The covariables, chemical components (Table I), and the factors, forage family (legume or grass) and method of conservation (fresh or conserved), were used to improve or to correct the OMD predictions.

The following model was used to find prediction equations:

(1) $OMD = \beta_0 + \beta_1 * technique_j + \beta_2 * covariable_k + factor_l + \varepsilon_{jkl}$

$Technique_j$ = DM or OM digestibility measured by the pepsin-cellulase technique, the *in situ* nylon bag technique, the gas production technique and the technique of Tilley and Terry or chemical components; $covariable_k$ = chemical components; $factor_l$ = forage family (legume or grass), method of conservation (fresh or conserved); β_{0to2} = regression coefficients; ε_{jkl} = residual error, supposed to be normally distributed with zero mean and constant (residual) standard error (RSE). *P* values of the equations and estimates were lower than 0.05.

The difference between the observed and predicted OMD was calculated as the mean square prediction error (MSPE), according to Bibby and Toutenberg (1977):

(2) $MSPE = 1/n \sum(O-P)^2$

O is the observed value and P is the predicted value and n is number of observations. The square root of MSPE expressed as a percentage of the observed mean is used as a measure of the prediction error (PError). MSPE was divided into the error in central tendency (bias), error due to deviations of the regression slope from one and error due to disturbances (unexplained variation) (Bibby and Toutenburg, 1977).

Table III. Effect (*P)* of dry matter intake (DMI, gram per kg metabolic body weight ($g \cdot kg^{-0.75}$ BW)) on OM digestibility in the total tract of forages.

Database		DMI		DMIcor[a]	
database 12[c]	All forages (n=12)	NS[b]		-	
	Grasses (n=6)	NS[b]		-	
	Legumes (n=6)	NS[b]		-	
12 with ad libitum[c]	All forages (n=12)	NS[b]		NS[b]	
	Grasses (n=6)	NS[b]		NS[b]	
	Legumes (n=6)	NS[b]		NS[b]	
database 37[c]	All forages (n=37)	NS[b]		NS[b]	
	Grasses (n=20)	NS[b]		$P < 0.01$	
	Legumes (n=17)	NS[b]		NS[b]	
database 61[c]	All forages	$P < 0.01$	(n=58)	$P < 0.001$	(n=48)
	Grasses	$P < 0.005$	(n=46)	$P < 0.001$	(n=36)
	Legumes	NS[b]	(n=12)	NS[b]	(n=12)

[a] Correction for seasonal influences on DMI, as described in Dulphy et al (1999a).
[b] NS = non significant : $P > 0.05$.
[c] Database 12 with (restricted feeding); database with same forages as database 12 but measured as in database 37 (12 with *ad libitum* feeding); database 37 (*ad libitum*); database 61 (*ad libitum*).

3. Results

3.1. General

Database 61 showed a broad range of quality, DM intake (Table I), OMD and digestibility values determined with alternative techniques (Table II). This database also included forages of low quality, resulting in a lower mean DMI than database 37. Mean DMI of database 12 was lower than that of database 37, because experiment 1 was performed with sheep fed restricted.

DMI had no effect on OMD using the same 12 forages from the databases 12 and 37 (Table III), measured respectively with restricted feeding and with *ad lib* feeding. Increased DMI had a positive effect on OMD of grasses in both other databases (Table III). DMI corrected for seasonal influences, had a significant effect on OMD predictions by some alternative techniques using database 61 (Table IV). However this effect was negative when using pepsin-cellulase and effective degradable DM and was positive when using undegradable DM, DM residue after 72 h of *in situ* incubation and gas production after 20 h. This significant effect disappeared when OMD predictions included chemical components, gas production parameter B2 or when predictions were separated for grass and legume (forage family). Forage family, CP, NDF and B2 were correlated with corrected DMI of grasses: correlation coefficients were between 0.61 and 0.68.

3.2. Prediction of OMD digestibility

Most RSE of the OMD predictions by alternative techniques, using database 37, were lower than 40 (g $\cdot kg^{-1}$ of OM intake) compared to the OMD mean (632 g $\cdot kg^{-1}$ of OM intake) and R^2 was low and very variable (Table IV). The PError of the validation of the OMD predictions with database 61 (Table IV) and database 12 (Table VI) were lower than 10 %, but lowest for the latter. But these validations differed in contributions of errors to the MSPE, errors due to bias, to regression and to disturbance (random deviation).

OMD predictions from CP, NDF and ADF were not significant ($P > 0.05$) and did not improve when more chemical components, DM or ash were included in the prediction equation, as in the Dutch feed evaluation system (CVB, 2001).

3.2.1. The in situ nylon bag technique for digestibility prediction

Values of OMD, calculated with parameters obtained by the nylon bag technique, were similar (regression coefficient = 0.994) to the values of effective degradable DM using a passage rate of 3 % h^{-1}. Using this kp, RSE and PErrors decreased and a negative DMI effect on the OMD prediction disappeared, when grass and legumes were considered separately or when CP was included in the prediction (Table IVa

Table IVa. Predictions of OMD by regression analysis of OMD with forage digestibility measured with the *in situ* nylon bag technique using database 37 and validation of these predictions using database 61 and the effect of DMI on these OMD predictions.

Technique (T)	Regression with database 37			Validation with database 61							
					PError[d]	MSPE[d] due to (%)			DMI cor[a]	
	Predictions	R^2	RSE[d]	n	(%)	Bias	Regression	Disturbance	*P*	n
In situ, effective degradable DM										
kp = 3.0										
Constant = 0	0 + 0,994 * T	-	50	39	7.4	0.0	56.0	44.0	< 0.05	36
+ L/G[b]	0 + 0.576 * T + 287 (grass) + 249 (legume)	0.63	33	39	4.0	4.5	48.1	47.4	NS[C]	36
Constant ≠ 0	351 + 0,445 * T	0.38	36	39	6.4	0.0	41.5	58.5	< 0.05	36
+ CP	275 + 0,696 * T – 0,621 * CP	0.62	33	39	5.2	3.5	22.4	74.1	NS[C]	36
kp = 4.5	417 + 0.366 * T	0.30	38	39	7.4	0.4	38.4	61.2	< 0.05	36
kp = 6.0	448 + 0.333 * T	0.30	39	76	7.8	0.3	35.3	64.4	< 0.05	36
kp = kd										
Constant = 0	0 + 1,134 * T	0.24	40	39	5.6	0.8	15.5	83.7	NS[C]	36
Constant ≠ 0	270 + 0,652 * T	0.53	31	39	6.4	0.1	34.9	65.0	NS[C]	36
DM residue after 72 h	757 - 0.412 * T	0.42	35	39	7.9	0.1	37.7	62.2	< 0.001	36
Undegradable DM	734 – 0.518 * T	0.46	33	39	9.3	1.7	19.4	78.9	< 0.001	36

[a] Correction for seasonal influences on DMI, as described in Dulphy et al (1999a).
[b] L/G = legumes or grasses.
[c] NS = non significant: *P* > 0.05.
[d] RSE = residual standard error; PError = prediction error; MSPE = mean square prediction error.

and 6a). However different predictions for grasses and legumes resulted in a higher error due to bias in the validation with database 12.

When kp was assumed to be equal to kd, rather than 3 % h^{-1}, predictions and validations were improved and also DMI had no effect on this prediction. When kd was multiplied by 0.4, as proposed by Van Vuuren (1993), OMD prediction had a higher PError when validated with database 12.

Even DM residue after 72h of incubation or undegradable DM had a good OMD prediction, although the effect of DMI did not disappear when chemical components or the factor forage family were included in the prediction.

3.2.2. Pepsin-cellulase technique for digestibility prediction

The negative DMI effect on OMD prediction by the pepsin-cellulase technique disappeared when CP, NDF or forage family were introduced into the predictions (Table IVb). PError of the validation with database 12 (Table VIa) decreased with CP and NDF, but increased with forage family due to bias.

3.2.3. Gas production technique for digestibility prediction

Lowest PError in OMD prediction by gas production after 20 h was found when this prediction included CP or NDF (Table IVb and VIb), whereas DMI had no effect. When using only parameters (A, B and C) from the three phases in the gas production profiles to predict OMD, gas production after 20 h plus B2 gave good results and a positive DMI effect on OMD prediction by gas production disappeared when B2 was included in contrary to C2.

3.2.4. In vitro technique according to Tilley and Terry for digestibility prediction

Low RSE and PError in OMD regression and validation respectively were also observed using the technique of Tilley and Terry (1963) (Table V and VIb). The OMD prediction did not improve when the values were corrected with *in vivo* values and when grasses and legumes had different prediction equations. No DMI effect on prediction of OMD of all forages, grasses or legumes was observed using database 37.

4. Discussion

4.1. Chemical composition and DMI effect

Chemical components were less accurate in predicting OMD than *in situ* and *in vitro* alternative techniques. Other studies (Aufrère and Michalet-Doreau, 1988; Steg et al., 1990) also showed that *in vitro* digestibility determinations using enzymes or

Table IVb. Predictions of OMD by regression analysis of OMD with forage digestibility measured with the pepsin-cellulase technique and the gas production technique, using database 37 and validation of these predictions using database 61 and the effect of DMI on these OMD predictions.

Technique (T)	Regression with database 37				Validation with database 61					
					PError [d]	MSPE [d] due to (%)			DMI cor [a]	
	Predictions	R^2	RSE [d]	n	(%)	Bias	Regression	Disturbance	*P*	n
Pepsin – Cellulase										
	412 + 0,372 * T	0.34	37	57	7.5	3.8	21.3	74.9	< 0.005	44
+ L/G [b]	0 + 0,679 * T + 263 (grass)									
	+ 197 (legume)	0.71	24	57	4.6	12.6	0.2	87.2	NS [c]	44
+ CP	394 + 0,512 * T – 0.484 * CP	0.42	35	57	7.7	2.7	17.3	80.0	NS [c]	44
+ NDF	- 73 + 0,770 * T + 0.449 * NDF	0.52	31	57	6.9	0.0	4.6	95.4	NS [c]	44
Gas production technique										
Degradable OM	0 + 0.969 * T	< 0.1	45	54	8.2	0.0	31.1	68.9	NS [c]	41
	312 + 0.496 * T	0.57	30	54	7.1	0.5	9.4	90.2	NS [c]	41
Gas production 20h	0 + 3.519 * T	-	55	54	9.2	1.0	40.9	58.1	< 0.05	41
	364 + 1.506 * T	0.55	31	54	7.6	2.2	11.9	85.9	< 0.05	41
+ CP	300 + 1.616 * T + 0,332 * CP	0.60	29	54	6.6	2.8	4.3	92.9	NS [c]	41
+ NDF	432 + 1.483 * T - 0,116 * NDF	0.58	30	54	6.9	5.1	13.5	81.4	NS [c]	41
+ B2	391 + 1.474 * T – 0.244 * B2	0.54	31	54	7.6	4.4	11.5	84.1	NS [c]	41
+ C2	145 + 1.232 * T + 10.4 * C2	0.66	27	54	8.3	0.8	6.9	92.3	< 0.05	41

[a] Correction for seasonal influences on DMI, as described in Dulphy et al (1999a)
[b] L/G = legumes or grasses
[c] NS = non significant: $P > 0.05$
[d] RSE = residual standard error; PError = prediction error; MSPE = mean square prediction error.

rumen fluid were consistently superior to chemical characteristics in predicting *in vivo* digestibility. However chemical components can optimise the OMD prediction by alternative techniques (Chenost et al., 2001; De Boever et al., 1999), not only by their contributions to digestibility but also by their effect on DMI. Chemical components are related to DMI, as NDF is well related to gastrointestinal fill (Van Soest, 1982). DMI effect was positively related with CP and negatively related with NDF.

The effect of forage family on the OMD predictions was due to the difference in chemical compostion between grasses and legumes. Grasses have higher cell wall contents, but lower lignin and CP contents than legumes (Merchen and Bourquin, 1994). Therefore the DMI effect on OMD was mainly due to the grasses because of the higher cell wall content and because grasses and legumes differ in functional specific gravity and thus in rumen passage rate (Hooper and Welch, 1985). Rumen passage rate and DMI do not only affect each other but also they affect ruminal digestion, which is the main part of OMD (Djouvinov and Todorov, 1994).

A positive or negative influence of DMI on OMD predictions by alternative techniques depends on two phenomena. First a higher DMI increases passage rate and thus decreases the amount of OM digested in the rumen and OMD, although this effect is limited with forage diets fed above maintenance (Galyean and Owens, 1991; Chilliard et al., 1995). Alternative techniques did not take into account this dynamic DMI effect and effective degradable DM was calculated with a constant passage rate. Secondly *in vivo* and alternative techniques differ in capacity of OM degradation.

Table V. Predictions of OMD by regression analysis of OMD with forage digestibility measured with the technique of Tilley and Terry with measured and corrected values using database 37 and the effect of DMI on these OMD predictions.

Technique (T)	All or Grass (G) or Legume (L)	Regression with database 37							*P*
						MSPE [c] due to (%)			
		Predictions	R^2	RSE [c]	n	Bias	Regres-sion	Distur-bance	DMI-cor[a]
Tilley & Terry									
measured	All	0 + 0.966 * T	0.71	24	37	0.0	4.7	95.3	NS[b]
	G	0 + 0,959 * T	0.79	25	20	0.1	0.0	99.0	NS[b]
	L	0 + 0,977 * T	0.49	24	17	0.4	16.6	83.0	NS[b]
	All	76 + 0.850 * T	0.72	24	37	0.0	0.0	100	NS[b]
Corrected	All	0 + 0.997 * T	0.35	37	37	0.8	57.4	41.8	NS[b]
	All	266 + 0.580 * T	0.72	24	37	0.0	0.0	100	NS[b]

[a] Correction for seasonal influence on DMI, as described in Dulphy et al (1999a)
[b] NS = non significant: *P* > 0.05
[c] RSE = residual standard error; MSPE = mean square prediction error.

Table VIa. Validations with database 12 of the OMD predictions from database 37 using the *in situ* nylon bag technique and the pepsin-cellulase technique.

Technique (T)		All or Grass (G) or Legume (L)	Regression with database 37	Validation with database 12			
			Grasses n = 20; Legumes n = 17	Grasses n = 6; Legumes n = 6			
				PError [b]	MSPE [b] due to (%)		
			Predictions	(%)	Bias	Regression	Disturbance
In situ, effective degradable DM							
kp = 3.0	Constant = 0	All	0 + 0,994 * T	5.2	8.1	35.2	56.7
	+ L/G[a]	All	0 + 0.576 * T + 287 (grass)				
			+ 249 (legume)	4.7	25.8	3.8	70.4
	Constant ≠ 0	All	351 + 0,445 * T	4.5	1.4	22.9	75.7
	+ CP	All	275 + 0,696 * T – 0,621 * CP	2.2	8.4	31.8	59.8
kp = 4.5		All	417 + 0.366 * T	4.9	1.7	16.3	82.0
kp = 6.0		All	448 + 0.333 * T	5.1	1.3	13.0	85.7
kp = kd:	Constant = 0	All	0 + 1,134 * T	4.9	14.4	23.3	62.3
	Constant ≠ 0	All	270 + 0,652 * T	4.2	5.4	10.9	83.7
DM residue after 72 h		All	757 – 0.412 * T	6.6	0.9	0.6	98.5
Undegradable DM		All	734 – 0.518 * T	6.7	1.1	0.0	98.9
Pepsin – Cellulase							
		All	412 + 0,372 * T	6.1	0.1	0.9	99.0
	+ L/G[a]	All	0 + 0,679 * T + 263 (grass)				
			+ 197 (legume)	8.4	38.4	9.1	52.5
	+ CP	All	394 + 0,512 * T – 0.484 * CP	5.1	0.0	9.1	90.9
	+ NDF	All	- 73 + 0,770 * T + 0.449 * NDF	5.3	0.2	18.5	81.3

[a] L/G = legumes or grasses

[b] PError = prediction error; MSPE = mean square prediction error.

The lower PErrors in the validation with database 12 compared to the validation with database 61, were also due to a DMI effect on OMD. The restricted feeding in database 12 resulted in reduced variation of OMD and thus in lower errors in the OMD prediction by alternative techniques.

4.2. In situ nylon bag technique

The nylon bag technique was performed with cows fed on a standard ration, whereas OMD was determined in sheep. Caution is needed when translating the results from one species to the other, as these species can differ in ruminal passage rates and feed digestion (Colucci et al., 1990; Dulphy et al., 1994; Poncet et al., 1995). Also degradation characteristics measured with the nylon bag technique may differ between cows and sheep (Šebek and Everts, 1999).

Nevertheless the results from the nylon bag technique were as accurate as the results from the other techniques. Observed OMD values and predicted OMD values from the *in situ* technique were similar using a kp of 3 % /h. This passage rate of DM in the total digestive tract was close to the values found by Luginbuhl et al. (1994), investigating whole-tract digesta kinetics in steers fed on coastal bermuda hay at four levels of intake.

When kp equalled kd, the predictions improved because degradation rate is positively related to OMD (Bosch et al., 1992; Fonseca et al., 1998) and probably to passage rate. Van Vuuren (1993) hypothesised that in roughage of low quality and with a slow rate of degradation, the increase in functional specific gravity will be more gradual, and thus, the slower rate of degradation is compensated by a slower rate of passage. Van Vuuren observed a better prediction of theoretical fermentable OM (Tamminga et al 1994) by the *in situ* technique using a passage rate as a function of the degradation rate.

OMD can also be predicted by the DM fraction degraded (Fonseca et al., 1998) or undegraded after 72 h of incubation.

4.3. Pepsin-cellulase technique

Different techniques with cellulase to measure digestibility have been developed, but their usefulness in forage evaluation will ultimately depend on the reliability and consistency of the predictive equations derived for *in vivo* digestibility (Jones and Theodorou, 2000). In this study the pepsin-cellulase technique developed by Aufrère and Demarquilly (1989) met these claims. Moreover this technique showed similar accuracy as the other alternative techniques. Other forage studies had different results. Some studies (De Boever et al., 1988; Givens et al., 1989) observed more accuracy with techniques using rumen fluid than techniques using cellulase, in contrast with other forage studies (Aufrère and Michalet-Doreau, 1988; Steg et al., 1990;Givens et al., 1990).

Table VIb. Validations with database 12 of the OMD predictions from database 37 using the gas production technique and the technique of Tilley and Terry with measured and corrected values.

Technique (T)	All or Grass (G) or Legume (L)	Regression with database 37	Validation with database 12			
		Grasses n = 20; Legumes n = 17	Grasses n = 6; Legumes n = 6			
			PError [a]	MSPE [a] due to (%)		
		Predictions	(%)	Bias	Regression	Disturbance
Gas production technique						
Degradable OM	All	0 + 0.969 * T	5.3	0.5	26.5	73.0
	All	312 + 0.496 * T	4.8	2.0	10.1	87.9
Gas production 20h	All	0 + 3.519 * T	9.4	4.5	60.3	35.2
	All	364 + 1.506 * T	5.7	4.2	0.2	95.6
+ CP	All	300 + 1.616 * T + 0,332 * CP	5.0	5.4	4.5	90.1
+ NDF	All	432 + 1.483 * T - 0,116 * NDF	4.9	2.8	1.9	95.4
+ B2	All	391 + 1.474 * T – 0.244 * B2	5.4	2.7	0.0	97.3
+ C2	All	145 + 1.232 * T + 10.4 * C2	5.0	3.9	0.2	95.9
Tilley and Terry technique						
Measured	All	0 + 0.966 * T	5.0	6.5	3.6	90.0
	G	0 + 0,959 * T	4.0	9.0	2.0	89.0
	L	0 + 0,977 * T	4.5	2.4	30.1	67.5
	All	76 + 0.850 * T	5.1	4.6	8.9	86.5
Corrected	All	0 + 0.997 * T	6.2	14.3	5.6	80.1
	All	266 + 0.580 * T	5.9	6.4	4.7	88.9

[a] PError = prediction error; MSPE = mean square prediction error.

4.4. Gas production technique

As with the Tilley and Terry technique, degradable OM measured with the gas production technique had low prediction errors without including chemical components. The differences in equations between the Tilley and Terry technique and the gas production technique were due to the differences in incubation time (48 h and 72 h respectively), dilution of rumen fluid with buffer (1:4 v/v and 1:3 v/v respectively) and because rumen incubation with the Tilley and Terry technique was followed by incubation with acid pepsin.

Gas volume may be the best indicator of the rumen apparent digestibility (Blümmel and Ørskov, 1993), which mainly affects OMD. Gas production as a measure for OM degradation was often observed as promising, when compared with the *in situ* nylon bag technique (Khazaal et al., 1993; Cone et al., 1998; Rymer and Givens, 2002). With OMD as reference, Chenost et al. (2001) concluded that the gas production technique can be as accurate as the pepsin-cellulase technique.

OMD prediction by gas production improved when chemical components were included in the prediction. CP had an effect on gas production after 20 h of incubation, because protein fermentation influences gas production (Cone and Van Gelder, 1999). This CP effect was also observed by Chenost et al. (2001) on gas production after 12 or 24 h of incubation. Effect of fibre on the gas production after 20 h of incubation depends on the maturity of the forages. Because CP, NDF and B2 were related to DMI, a positive DMI effect on these OMD predictions disappeared when they were included in the predictions.

4.5. The Tilley and Terry technique

The *in vitro* technique of Tilley and Terry (1963) has a good predictive value, similar to or better than the other techniques. It is often used as the reference technique for other techniques to evaluate OM digestion, such as the pepsin-cellulase-technique (De Boever et al., 1988; Givens et al., 1989 and 1990; Aufrère and Michalet-Doreau, 1988), the *in situ* nylon bag technique and the gas production technique (Cone et al., 1998 and 1999; Khazaal et al.,1993).

The Tilley and Terry technique had low prediction errors and chemical components did not improve the predictions. Only databases 12 and 37 were determined with this technique, thus a DMI effect on its OMD prediction using database 61 was not tested. Nevertheless DMI had no effect when grasses and legumes were separated in different predictions by this technique (Table IVb), whereas DMI had an effect on OMD of grasses using database 37.

Measured values were at least as good as values corrected to *in vivo* values.

4.6. Ranking of the alternative techniques in predicting OMD

A ranking of the four techniques compared in predicting OMD should be based on their validation with database 12 without a DMI effect on the prediction in database 61. The lowest PErrors of the techniques using the validation of database 12 were: 2.2 % (*in situ*, kp=3 inclusive CP), 4.9 or 5.0 % (gas production after 20 h inclusive NDF or CP), 5.0 % (measured values from technique of Tilley and Terry) and 5.1 % (pepsin-cellulase inclusive CP). The same order could be found when comparing the validations with database 61. Thus the *in situ* technique showed best result. Although this technique needs fistulated animals, it needs less animals, it can investigate more feed samples per animal and it is less time consuming than the measurement of *in vivo* OMD. Moreover the *in situ* technique provides useful additional information of kinetics and degradation parameters for different chemical components.

Nevertheless *in vitro* techniques can also be used as good alternatives for predicting OMD. These techniques have a high accuracy, a good repeatability and a similar potency to predict OMD. When chemical components were included in the estimation of OMD the accuracy improved and the repeatability remained good. However, with more variables in a prediction the chance of a lower repeatability should be taken into account.

Other reasons for choosing a technique to predict OMD will also be important, because the four techniques slightly differ in potency to predict OMD. The reasons can be costs, time, animal welfare, experience, additional information and already existing databases. Of the four techniques, the *in situ* technique is most expensive, time consuming and it reduces animal welfare. The gas production technique delivers also dynamic parameters for OM degradation, but not for the degradation of chemical components. The gas production technique and the two other *in vitro* techniques do not differ much in costs and time. The gas production technique and the technique of Tilley and Terry (1963) need rumen-fistulated animals, which are not necessary for enzymatic techniques. Another advantage of enzymatic techniques can be that they will have the best reproducibility of all four techniques, because it used enzymes and not the rumen or rumen fluid of living animals.

5. Conclusions

The prediction by the *in situ* nylon bag technique including crude protein showed the highest accuracy in the validations, although all four techniques had good results in predicting OMD.

Also other reasons for choosing a technique to predict OMD will be important. These reasons can be costs, time, animal welfare, experience, additional information, reproducibility and already existing databases.

Because ruminal OM digestion is the main part of OMD and is a measure for energy necessary for microbial protein synthesis in the rumen, it will be interesting also to investigate if the alternative techniques can predict OM digested in the rumen.

Chapter 3

Comparison of *in situ* and *in vitro* techniques to predict *in vivo* fermentable organic matter of forages in ruminants

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Comparison of *in situ* and *in vitro* techniques to predict *in vivo* fermentable organic matter of forages in ruminants

Abstract

The objectives of this study was to investigate if *in vivo* determined fermentable organic matter (FOM) can be predicted from FOM measured with *in situ* and *in vitro* techniques (measured FOM) and from FOM calculated using OM total tract digestibilty (OMD), which was measured with *in situ* and *in vitro* techniques (calculated FOM). This calculation was according to the French and Dutch protein evaluation systems. Investigated were 12 forages: fresh and conserved forms of lucerne, red clover, orchard grass and perennial ryegrass. OM truly digested in the rumen (OMTDR) was regarded as *in vivo* FOM and was calculated from digesta flows in sheep with cannula in the rumen, duodenum and ileum. Digesta flows were measured using ^{51}Cr-EDTA and ^{103}Ru-Phenanthrolin. OMTDR was measured in sheep fed restricted and OMD was measured with sheep fed restricted and ad libitum. OMTDR was related with the chemical composition and with measured and calculated FOM of the 12 forages using the *in situ* nylon bag technique and three *in vitro* techniques. An enzymatic technique using pepsin and cellulase, the technique of Tilley and Terry and the gas production technique were used. Comparing these techniques concerning OMTDR prediction from measured FOM, the *in situ* technique gave good results. The gas production after 20 h incubation was best related with OMTDR (R^2 = 0.74). The relationship between OMTDR and gas production after 20 h improved when fresh and conserved forages were considered separately (R^2 = 0.90). OMTDR was well predicted by calculated FOM when using the *in situ* technique, the gas production technique and the technique of Tilley and Terry (R^2 = 0.76 to 0.80). OMTDR predictions from measured and calculated FOM had similar accuracy, although OMTDR prediction from calculated FOM was a validation and OMTDR prediction from measured FOM was a regression. The *in situ* and gas production technique gave best results as they approached rumen dynamics most closely, but these dynamics lead to high values of SD and thus reduced the repeatability of these techniques.

Keywords: nylon bag / gas production / Tilley and Terry / pepsin / cellulase / FOM

1. Introduction

Fermentable organic matter (FOM) of forages is a good measure for energy production in the rumen, an important factor for determining the synthesis of microbial protein in the rumen and consequently profitable for the animal. *In vivo*

measurements of FOM are expensive and laborious and reduce animal welfare. In protein evaluation systems (Vérité et al., 1987; Tamminga et al., 1994) FOM is calculated from OM total tract digestibility of forages (OMD). In the past 40 years great efforts were done to develop alternative techniques to measure FOM.

The most frequently used alternatives are the *in situ* technique and the gas production technique. Also other techniques to measure OMD, such as the pepsin-cellulase technique (Aufrère and Demarquilly, 1989) and the *in vitro* technique according to Tilley and Terry (1963), can be used to estimate FOM. These 4 techniques are well correlated to OMD (Aufère and Michalet-Doreau, 1988; Tilley and Terry, 1963; Menke and Steingass, 1988; Fonseca et al., 1998) and have been compared with each other (Givens et al., 1989; Blümmel and Ørskov, 1993; Chenost et al., 2001; Cone et al., 1999). However these comparisons were usually done with only two of these techniques and different procedures for the same techniques were used. Evaluations of *in situ* and *in vitro* techniques as predictors of FOM, measured *in vivo*, are scarce. The *in situ* technique has been related to *in vivo* FOM in a study using a variety of feedstuffs (Arieli et al., 1998). Rymer and Givens (2002) compared patterns of rumen fermentation with *in situ* degradability and gas production profiles.

To relate OM degradation, measured by the techniques mentioned above, with *in vivo* measured FOM is more difficult than to relate them with *in vivo* measured OMD. Firstly more *in vivo* OMD data are available, because measuring OMD is easier than measuring FOM. Secondly FOM depends on rumen dynamic processes and OMD depends on OM digestion in the different compartments of the total digestive tract, which can compensate each other. Differences between these alternative techniques will probably be more pronounced when related to FOM than related to OMD. Enzymatic techniques are more robust than incubation of forages in rumen fluid as in the *in situ* method and some other *in vitro* techniques. Comparing *in vitro* techniques using rumen fluid, the technique according to Tilley and Terry (1963) is not dynamic and therefore has less variable results than the gas production technique.

Alternative techniques most widely used in France and the Netherlands are the pepsin-cellulase technique (Aufrère and Demarquilly, 1989), the *in situ* technique (Michalet-Doreau et al., 1987), the gas production technique (Cone et al., 1996a) and the *in vitro* technique according to Tilley and Terry (1963). In this study these techniques are evaluated in their prediction of OM truly digested in the rumen (OMTDR), which was regarded as *in vivo* FOM. Firstly OMTDR prediction from FOM measured with these alternative techniques was investigated. Secondly a choice was made in predicting OMTDR between this measured FOM and calculated FOM, which is FOM calculated according to French and Dutch protein evaluation systems (Vérité et al., 1987; Tamminga et al., 1994) from OMD measured with the alternative techniques.

2. Materials and Methods

2.1. Forages

OM digested in the rumen and in the total tract of 12 forages were determined. These 12 forages were fresh form, silage and hay of Lucerne (*Medicago sativa*), red clover (*Trifolium pratense*), orchard grass (*Dactylis glomerata*) and perennial ryegrass (*Lolium perenne*). Red clover haylage, a wilted forage wrapped in bales and with a dry matter content of about 500 g/kg forage, was made in stead of red clover hay because of wet harvest conditions.

2.2. *In vivo* measurement of OM degradation in the rumen and total tract

OMTDR is the OM apparently digested in the rumen (OMADR) inclusive bacterial OM entering the duodenum. OMADR is the difference between OM intake and OM entering the duodenum. Bacterial OM entering the duodenum was calculated from the duodenal flow of bacterial N assuming that the N/OM ration in bacteria was 10 % (Clark et al., 1992). The duodenal flow of OM, bacterial N and OMD were measured in an *in vivo* experiment with cannulated sheep fed restricted, using ^{51}Cr-EDTA and ^{103}Ru-Phenanthrolin as flow markers and ^{15}N as microbial marker (Gosselink et al., 2003a). This study contained only the *in vivo* measurement of the digestion of the six legumes.

The six grasses were investigated with the same methodology as used for the legumes, except for fresh perennial ryegrass. This ryegrass was not investigated with the double marker measurement, but with only one marker, Ru-phenantrolin (non radioactive). When comparing these two measurements using the other 11 forages, the difference in the duodenal flow of OM and non ammonia N (NAN) was not significant. But the flow of bacterial N was about 5.4 % (range = 0.75 to 8 %) lower for the one marker measurement. This percentage was used in the determination of the duodenal flow of bacterial OM for fresh perennial ryegrass.

In another experiment, OMD of the 12 forages was measured in sheep with ad libitum feeding (Gosselink et al., 2003b) .

2.3. *In situ* and *in vitro* measurements of FOM

In situ and *in vitro* measurements of FOM were performed by measuring OM and DM degradation of the 12 forages with the *in situ* technique (Michalet-Doreau et al., 1987) and 3 *in vitro* techniques. The *in vitro* methods were the pepsin-cellulase technique (Aufrère and Demarquilly, 1989), the gas production technique (Cone et al., 1996a) and the two-stage *in vitro* technique according to Tilley and Terry (1963).

Table I. Dry matter (DM, g ·kg^{-1}), chemical composition (g ·kg^{-1} DM) and rumen escape protein determined with the *in situ* technique (REP, g ·kg^{-1} DM) of the 12 forages.

Forage	Method of conservation	DM	Ash	CP	NDF	ADF	REP
Lucerne	fresh	162	138	198	498	346	43.4
	silage	212	98	182	438	328	32.2
	hay	861	99	171	560	379	54.0
Red clover	fresh	127	120	168	492	348	18.5
	silage	171	92	166	478	343	28.3
	haylage	524	108	128	475	352	25.9
Orchard grass	fresh	193	80	116	676	360	33.9
	silage	217	71	126	614	343	20.4
	hay	852	70	110	697	376	36.7
Lolium perenne	fresh	182	98	91	620	366	17.5
	silage	191	92	101	578	371	11.8
	hay	873	96	91	632	382	25.5

Table II. Fermentation quality of the 4 silages and 1 haylage from table 1: pH, ammonia (NH_3: g kg^{-1} of DM) and fermentation products (g·kg^{-1} of DM): HL = lactic acid, HAc = acetic acid, HP = propionic acid, HB = butyric acid and ethanol.

Forage	Method of conservation	pH	NH_3	HL	HAc	HP	HB	ethanol
Lucerne	silage	4.03	2.24	45.6	29.6	0.30	0.00	5.79
Red clover	silage	3.97	1.93	69.4	23.6	0.73	0.20	4.08
Red clover	haylage	5.11	1.71	24.3	3.6	0.96	0.75	2.35
Orchard grass	silage	3.93	1.21	78.5	14.9	0.15	3.09	3.42
Lolium perenne	silage	4.13	0.66	92.9	19.1	2.15	0.40	17.5

2.3.1. *In situ* technique

The method of sample preparation for measurement of DM degradation with the *in situ* technique was described by Dulphy et al. (1999b). The procedure of the measurement was according to Michalet-Doreau et al. (1987) and the data were fitted according to the method of Ørskov and McDonald (1979). Effective degradable DM was calculated using different passage rates. A passage rate of DM in the total tract of 3%/h showed best results in predicting OMD (Gosselink et al., 2003b). A ruminal passage rate of DM of 4.5 %/h is used in the Netherlands (Tamminga et al., 1994) and 6 %/h is used in France (Vérité et al., 1987). In the calculations also a passage rate (kp) equal to rumen degradation rate (kd) was used. Kp as function of

kd improved the prediction of FOM, calculated as in the Dutch protein evaluation system (Van Vuuren, 1993). Each forage was incubated in two series for each incubation period in three cows, receiving a ration with 70 % hay and 30 % concentrate.

2.3.2. Pepsin-cellulase technique

The enzymatic technique to measure DM degradation was the pepsin-cellulase technique developed by Aufrère (1982), including the use of 0.1N HCl (Aufrère and Demarquilly, 1989). DM degradation of each forage was determined in triplicate.

2.3.3. *In vitro* technique according to Tilley and Terry

OM digestibility was determined with the two-stage *in vitro* technique using rumen fluid and acid pepsin, as described by Tilley and Terry (1963). In this study measured values as well as values standardised with *in vivo* values according to the modification of Van der Meer (1986) were used in the comparisons. The determination of these measured and standardised values was done in duplicate.

2.3.4. Gas production technique

The forages were incubated in quadruplicate in the gas production technique, as described by Cone et al. (1996a). Gas production profiles were described with a three-phasic model (Groot et al., 1996), describing the gas production caused by fermentation of the soluble components (phase 1), the non-soluble components (phase 2) and microbial turnover (phase 3) (Cone et al., 1997). Each phase is described by three parameters: A (maximum gas production, ml/g OM), B (time at which 50% of the maximal gas production is reached in h) and C (parameter determining the shape of the curve, without dimension).

After 72 hours of incubation also OM degradation (as % of OM incubated) was determined by measuring the OM residue after filtering over a P1 glass crucible.

2.4. Calculation of FOM

FOM was calculated from OMD according to the French and Dutch protein evaluation system, (FFOM and DFOM: Vérité et al., 1987; Tamminga et al., 1994). Different origins of OMD were used: OMD measured in both *in vivo* experiments (restricted and ad libitum feeding) and OMD predicted from chemical components and alternative techniques. To calculate FFOM and DFOM from OMD, the content of fermentation products in silages and haylages, the amount of rumen escape protein and the content of crude fat from forages were used. The difference between FFOM and DFOM was caused by the different proportions of fermentation products in silage and haylage. In the French system 100 % and in the Dutch system 50 % of the

fermentation products were taken into account in calculating FOM. Fermentation products were determined according to Dulphy et al. (1975). Rumen escape protein of the 12 forages was measured using the *in situ* methodology as described in paragraph 2.4.2, calculated as in Michalet-Doreau et al. (1987) and corrected for microbial contamination of the residues in the nylon bags (Michalet-Doreau and Ould-Bah, 1989). It was assumed that the forages contained no starch and that crude fat in hay was 15 g/kg OM and crude fat in the other forages was 30 g/kg OM.

OMD predictions to calculate FFOM and DFOM were according to equations developed by Gosselink et al. (2003b), using the *in situ* technique, the pepsin-cellulase technique, the Tilley and Terry technique, the gas production technique and crude protein (CP). These OMD predictions are:

using the *in situ* technique and crude protein:

$$275 + 0.696 * \text{effective DM degradation} - 0.621 * \text{CP} \quad (1)$$

using the pepsin-cellulase technique and crude protein:

$$394 + 0.512 * \text{DM degradation} - 0.484 * \text{CP} \quad (2)$$

using the technique of Tilley and Terry:

$$0.966 * \text{OM degradation (measured in vitro values)} \quad (3)$$

using the gas production technique and CP:

$$300 + 1.162 * \text{gas production after 20 h} - 0.332 * \text{CP} \quad (4)$$

Table III. OM intake (OMI, g $\cdot d^{-1}$) and OM apparently digested in the rumen (OMADR), OM truly digested in the rumen (OMTDR) and OM digested in the total tract (OMD) as g $\cdot kg^{-1}$ of OMI, measured *in vivo* in sheep with restricted feeding or ad libitum feeding.

Forage	Method of conservation	Restricted feeding						Ad libitum feeding		
		OMI	OMADR	OMTDR	SD	OMD	SD	OMI	OMD	SD
Lucerne	fresh	1329	329	553	27.7	592	17.9	1506	600	13.0
	silage	1519	410	583	10.3	641	9.3	1335	617	8.1
	hay	1028	346	523	33.1	559	10.0	1144	564	17.4
Red clover	fresh	1141	518	739	31.6	725	16.7	1365	699	14.0
	silage	1206	458	624	24.5	682	13.0	1318	650	15.9
	haylage	1148	447	617	29.2	646	15.7	1159	650	15.9
Orchard grass	fresh	1226	419	609	17.6	629	8.3	1302	635	21.1
	silage	1214	383	556	27.7	612	16.9	1031	680	23.0
	hay	1078	357	519	13.2	558	22.5	1228	584	27.4
Lolium perenne	fresh	1191	519	691	23.54	671	15.2	1229	657	17.9
	silage	1195	420	609	23.5	658	14.2	1159	648	25.0
	hay	1162	407	589	27.9	635	5.4	1187	635	38.0

2.5. Chemical analysis

DM contents of feed and faeces were determined by drying at 80°C for 48 h, ash content was determined after 6 h at 550 °C and N was determined using the Kjehldahl method (AOAC, 1980).
Neutral detergent fibre (NDF) and acid detergent fibre (ADF) (Goering and Van Soest, 1970) were determined on samples dried at 60 °C.

2.6. Statistics

Statistical analyses were applied using Genstat (2002).
The factors, forage family (legume or grass) and method of conservation (fresh or conserved), and the covariables, chemical components, were also used to improve OMTDR predictions from FOM measured with alternative techniques. The following model was used to find OMTDR prediction equations:

(1) $OMTDR = \beta_0 + \beta_1 * technique_j + \beta_2 * covariable_k + factor_l + \varepsilon_{jkl}$

$Technique_j$ = DM or OM degradation measured by the pepsin-cellulase technique, the *in situ* technique, the gas production technique and the technique of Tilley and Terry or chemical components; $covariable_l$ = chemical components; $factor_l$ = forage family (legume or grass), method of conservation (fresh or conserved); β_{0to2} = regression coefficients; ε_{jkl} = residual error, supposed to be normal distributed with zero mean and constant (residual) standard error (RSE). P value of the equations and estimates were considered significant when lower than 0.05.
To evaluate the OMTDR predicted from different calculated FOM, R^2 and RSE of the relationship between OMTDR and calculated FFOM or DFOM were shown.
Mean square prediction error (MSPE) of OMTDR predictions from measured as well as calculated FOM were analysed. MSPE was calculated as the difference between the observed and predicted flows according to Bibby and Toutenberg (1977):

$$MSPE = 1/n \sum(O-P)^2$$

O is the observed value and P is the predicted value and n is number of observations. The square root of MSPE expressed as percentage of the observed mean is used as measure of the prediction error (PError). MSPE was decomposed into the error in central tendency (bias), error due to deviations of the regression slope from one and error due to disturbances (unexplained variation) (Bibby and Toutenberg, 1977).

Table IV. DM degradation (g $\cdot kg^{-1}$ of DM intake), determined with the *in situ* technique (kp = 6%/h) in cows (situ), determined with the pepsin-cellulase technique (p-cel), and OM degradation (g $\cdot kg^{-1}$ of OM intake) measured using the Tilley and Terry technique (T&T) or the gas production technique (gpdeg) and the gas production (ml $\cdot g^{-1}$ of OM) measured after 20 h (gpt): means and SD of the 12 forages from table I.

Forage	Method of conservation	situ	SD	p-cel	SD	T&T	SD	gpdeg	SD	gpt	SD
Lucerne	fresh	589	21.5	631	4.4	626	3.5	643	50.2	164	6.6
	silage	593	10.6	640	2.8	680	0.7	687	46.8	182	6.2
	hay	484	8.5	605	4.5	606	0.7	625	50.0	159	10.4
Red clover	fresh	709	14.2	696	7.1	700	2.1	761	45.7	218	8.0
	silage	639	12.1	649	3.3	675	0.0	718	50.4	203	10.1
	haylage	573	24.7	668	4.0	690	10.6	732	22.4	209	21.2
Orchard grass	fresh	491	33.0	516	3.8	642	1.4	696	64.1	183	14.1
	silage	496	6.7	556	2.8	609	1.4	714	44.1	188	9.8
	hay	407	16.8	462	2.9	672	1.4	661	33.4	165	10.5
Lolium perenne	fresh	532	23.5	587	3.3	728	1.4	766	83.6	222	16.6
	silage	551	49.7	583	6.2	684	3.5	727	40.5	210	7.6
	hay	506	16.8	546	8.7	673	6.3	701	30.2	192	17.4

3. Results

3.1. Database

The large variation in quality of the12 forages (Table 1 and 2) is favourable for finding predictions of FOM and for evaluating the accuracy of FOM predictions. This large variation resulted in a large range of OM digested in the rumen or in the total tract measured *in vivo* (Table 3). With restricted feeding OMD showed a larger range and lower SD (mean SD = 11.8) than ad libitum feeding (mean SD = 19.7) (Table 3).

The use of different forages also resulted in large ranges of degraded OM and DM, measured by *in situ* and *in vitro* techniques (Table 4). The results from the *in situ* technique and the gas production technique showed a higher SD than the other techniques. When the ratios of these SD and the means were compared with the ratios of SD and the means of OMTDR (mean ratio = 4.1), the ratio for the *in situ* technique (mean = 3.7) was lower and the ratio for the gas production after 20 h (gpt, mean = 6.0) was higher (Table 3 and 4).

The variation in calculated FFOM and DFOM values (Table 5) and their SD depended on the origin of OMD from which they were calculated, either OMD measured *in vivo* (Table 3) or OMD predicted from alternative techniques (Table 4).

3.2. OMTDR prediction from measured FOM

When evaluating all techniques in predicting directly OMTDR (Table 6), the gas production technique showed most potency. Gas production after 20 h incubation, inclusive as well as exclusive CP, had a good relationship with OMTDR. The highest R^2 and lowest RSE were found when the relationship between OMTDR and the gas production after 20 h was separated in relationships for fresh and conserved forages. The relationship between OMTDR and the gas production technique did not improve when other parameters from the gas production profiles were included. Therefore only the gas production after 20 h incubation was provided in the tables.

Table V. Fermentable OM (g $\cdot$kg^{-1} of OM intake) calculated from French (FFOM) and Dutch (DFOM) protein evaluation systems, using OMD measured *in vivo* in sheep fed restricted (R) or ad libitum (A) and OMD predicted from the *in situ* technique (situ), the pepsin-cellulase technique (p-cel), the technique of Tilley and Terry (T&T) or the gas production technique (gpt).

Forage	Method of conservation	FFOM or DFOM	R	A	situ	p-cel	T&T	gpt
Lucerne	fresh	both	512	530	521	542	509	530
	silage	FFOM	496	452	471	489	480	502
		DFOM	536	497	511	528	520	542
	hay	both	483	496	484	545	499	519
Red clover	fresh	both	674	657	648	618	617	641
	silage	FFOM	476	495	493	476	479	502
		DFOM	530	544	547	531	534	557
	haylage	FFOM	587	556	553	579	554	577
		DFOM	605	574	571	597	572	595
Orchard	fresh	both	562	572	547	535	532	553
grass	silage	FFOM	451	490	451	456	445	466
		DFOM	505	555	506	511	499	521
	hay	both	504	536	514	523	512	532
Lolium	fresh	both	622	610	605	601	614	637
perenne	silage	FFOM	469	459	466	455	458	481
		DFOM	541	532	538	527	530	553
	hay	both	591	492	592	586	576	598

Table VI. Predictions of OM truly digested in the rumen (g/kg OM intake) measured *in vivo* with sheep, by chemical composition or techniques from table 4, measuring DM or OM degradation, using residual error (RSE) and different contributions to the MSPE of the predictions.

Technique (T)	Predictions	R^2	RSE	MSPE due to (%) Bias	Regres-sion	Distur-bance
in situ						
kp = 3.0	0 + 0.955 * T	0.56	43	0.0	6.4	93.6
+ CP	0 + 1.160 * T – 0.937 * CP	0.78	30	0.1	7.7	92.2
kp = 4.5	0 + 1.027 * T	0.31	53	0.4	29.6	70.0
	233 + 0.632 * T	0.47	47	0.0	0.0	100
+ CP	220 + 0.912 * T – 1.090 * CP	0.78	30	0.0	0.0	100
kp = 6.0	0 + 1.087 * T	< 0.1	63	0.9	45.0	54.1
	295 + 0.560 * T	0.43	49	0.0	0.0	100
+ CP	293 + 0.856 * T – 1.176 * CP	0.78	30	0.0	0.0	100
kp = kd	0 + 1.094 * T	0.59	41	0.0	2.2	97.8
Pepsin-Cellulase						
	0 + 1.004 * T	-	65	0.3	27.7	72.0
Tilley & Terry						
Measured values	0 + 0.905 * T	0.48	46	0.1	7.8	92.1
Standardised values	0 + 0.903 * T	0.59	40	0.0	20.1	79.9
Gas production technique						
OM degradation after 72 h	0 + 0.857 * T	0.67	37	0.1	26.2	73.7
Gasproduction after 20 h	0 + 3.139 * T	0.74	33	0.1	11.4	88.5
+ MC[a]	0 + 2.406 * T + 176 (fresh) + 124 (conserved)	0.90	21	0.0	0.0	100
+ CP	0 + 2.815 * T + 0.418 * CP	0.80	29	4.7	0.5	94.8

[a] MC = method of conservation: fresh forage or conserved forage.

The *in situ* technique inclusive CP had also a high R^2 and a low RSE when related to OMTDR and exclusive CP the best results were found when kp was 3%/h or equal to kd (Table 6). The pepsin-cellulase technique, the technique of Tilley and Terry and OM degraded after 72 h incubation in the gas production technique had similar relationships with OMTDR, although in these relations MSPE was partly due to regression.

No chemical component was significantly related to OMTDR. When chemical components were combined or with the addition of DM or ash as variables, the relationships between chemical components and OMTDR did not improve.

3.3. OMTDR prediction from calculated FOM

Generally DFOM gave a better prediction of OMTDR than FFOM, when comparing R^2, RSE or the contribution of the regression to MSPE (Table 7), although DFOM and FFOM were close but lower than OMTDR. NDF had a positive effect on the OMTDR prediction by FFOM and DFOM, when they were calculated from *in vivo* OMD values measured with sheep fed ad libitum.

Using OMD predictions from *in situ* and *in vitro* techniques for the calculation of FFOM and DFOM, R^2 was lower and RSE was higher than when using *in vivo* OMD values. Of all techniques, the FFOM and DFOM calculation using OMD prediction from the *in situ* technique resulted in the best prediction of OMTDR. Nevertheless the gas production technique and the technique of Tilley and Terry showed also good results.

The prediction of OMTDR using FFOM or DFOM calculated from OMD measured with *in vitro* and *in situ* techniques was close to the prediction of OMTDR directly from FOM measured with *in situ* and *in vitro* techniques. Moreover using the measured *in vitro* values of the technique of Tilley and Terry, OMTDR prediction by the calculated DFOM had a higher R^2 and lower RSE than OMTDR prediction by FOM directly measured from the *in vitro* values .

3.4. Comparison of alternative techniques

OM degradation and gas production measured with the gas production technique were well related with DFOM calculated from *in vivo* OMD with restricted feeding (DFOM-R) and with OM degradation measured with the technique of Tilley and Terry and the *in situ* technique using kd equal to kp (Table 8). When kp was constant (kp = 6%/h), the results of the *in situ* technique were related with those of the pepsin-cellulase technique.

Table VII. Predictions of OM truly digested in the rumen (g/kg OM intake) measured *in vivo* with sheep, by the French and Dutch calculations of FOM (table 5: FFOM and DFOM) from OMD measured *in vivo* in sheep fed restricted (R) or ad libitum (A) and OMD predicted from crude fibre (CF), *in situ* technique (situ), pepsin-cellulase technique (p-cel), technique of Tilley and Terry (T&T) or the gas production technique (gpt), using residual error (RSE) and different contributions to the MSPE of the predictions.

Technique (T)	Predictions	R^2	RSE	MSPE due to (%)		
				Bias	Regres-sion	Distur-bance
FFOM-R	0 + 1.116 * T	0.36	51	0.4	33.2	66.4
	22.8 + 0.696 * T	0.53	44	0.0	0.0	100
DFOM-R	0 + 1.081 * T	0.82	27	0.0	1.6	98.4
FFOM-A	0 + 1.114 * T	0.22	57	0.3	24.7	75.0
+ NDF	383 + 0.736 * T – 0.317 * NDF	0.50	45	0.1	0.0	99.9
DFOM-A	0 + 1.078 * T	0.67	37	0.0	0.5	99.5
+ NDF	104 + 1.175 * T – 0.284 * NDF	0.78	30	0.3	0.1	99.6
FFOM-situ	0 + 1.132 * T	0.38	51	0.3	24.2	75.6
DFOM-situ	0 + 1.096 * T	0.80	29	0.0	2.4	97.6
FFOM-p-cel	0 + 1.121 * T	-	64	0.3	25.0	74.7
DFOM-p-cel	0 + 1.086 * T	0.52	45	0.0	1.8	98.2
FFOM-T&T	0 + 1.145 * T	0.34	53	0.2	20.3	79.5
DFOM-T&T	0 + 1.109 * T	0.76	32	0.0	7.3	92.7
FFOM-gpt	0 + 1.075 * T	0.33	52	0.3	11.8	87.9
DFOM-gpt	0 + 1.042 * T	0.74	32	0.0	25.3	74.7

4. Discussion

4.1. General

Measuring OMTDR is more difficult than measuring *in vivo* OMD because of methodology, costs and animal welfare. The measurement of OMTDR is also less accurate, as its SD is higher than that of OMD. Moreover OMD predictions from *in vitro* and *in situ* techniques are well validated (Gosselink et al., 2003b). The results of our research showed that the prediction from measured FOM was slightly superior to the prediction from calculated FOM.

4.2. OMTDR prediction from measured FOM

OMTDR predictions from the gas production technique and the *in situ* technique improved when a correction with CP was included. In the gas production technique protein fermentation influences gas production negatively (Cone and Van Gelder, 1999; Chenost et al., 2001). Notably with the *in situ* technique the inclusion of CP improved the accuracy of prediction considerably. *In situ* measurement of OM and DM degradation included all CP degraded in the rumen, whereas OMTDR misses CP degraded to ammonia that entered the duodenum. The regression coefficient of CP increased when kp increased, thus probably the CP fraction in the equation corrects the difference in degradable CP or other OM fractions flowing out of the rumen between OMTDR and effective degradable DM from the *in situ* technique.

The difference in OMTDR prediction by gas production between fresh and conserved forages was a result of differences in digestibility, because silage have a reduced soluble carbohydrate fraction and in hay the structural carbohydrate composition can be altered by leaf losses during harvesting (Merchen and Bourquin, 1994).

4.3. OMTDR prediction from calculated FOM

Using 50% of fermentation products to calculate DFOM resulted in better OMTDR prediction than using 100 % of fermentation products to calculate FFOM, although both underestimated FOM. FOM predictions by FFOM and DFOM, calculated from *in vivo* OMD values, improved when NDF was also included, due to the effect of fibre digestion in the large intestine on OMD. Probably this is more important in sheep than in cattle, notably in dairy cattle. The best OMD predictions from alternative methods, observed by Gosselink et al. (2003b) and used in this study, contained also a covariable (CP), except for the technique of Tilley and Terry.

Table VIII. Correlations (R^2) between methods from table VI and VII.

R^2	DFOM-R	*In situ* (kp=6)	*in situ* (kp=kd)	Pepsin-cellulase	Tilley & Terry	Gasproduction (20 h)	GPT OM degradation[a]
FFOM-R	0.85	< 0.1	< 0.1	< 0.1	0.31	0.22	0.25
DFOM-R		0.21	0.38	< 0.1	0.51	0.60	0.61
In situ (kp=6)			0.64	0.79	< 0.1	0.22	0.15
In situ (kp=kd)				0.34	0.21	0.53	0.56
Pepsin-cellulase					< 0.1	< 0.1	< 0.1
Tilley & Terry						0.56	0.51
Gasproduction (20 h)							0.94

[a] OM degradation measured with the gas production technique (GPT)

4.4. Rumen digestion and alternative techniques

Rumen digestion dynamics are important in the OMTDR prediction from measured as well as calculated FOM. The part of rumen digestion dynamics, which the *in situ* and the *in vitro* techniques were mimicking, is ruminal OM degradation. Another important part of rumen digestion dynamics is the ruminal OM passage rate. Only predictions based on the *in situ* technique takes passage rate into account, although feed evaluation systems use a constant rumen passage rate (Vérité et al., 1987; Tamminga et al., 1994). With a variable passage rate (kp = kd), OMTDR or OMD predictions from the *in situ* technique improved. However the impact of passage rate on OMTDR prediction was low when comparing different rates. This low impact was mainly the result of the limited effect of DM intake on ruminal passage rates and OM digestion when forages are fed above maintenance (Galyean and Owens, 1991; Chilliard et al., 1995).

4.5. Comparison of alternative techniques

Comparing the predicted results of the *in vitro* and *in situ* techniques showed that rumen digestion dynamics are also the base to compare these alternative techniques. The *in situ* technique showed a good correlation with the gas production technique when kp = kd and the *in situ* technique was related to the pepsin-cellulase technique when kp was constant. Dynamic parameters from the gas production technique and the *in situ* technique are related (Cone et al., 1998; Rymer and Givens 2002), although these relationships were moderate in this study; R^2 was 0.37 between *in situ* degradation rate and b in the second phase of the gas production profile. Using grass samples, Cone et al. (1999) observed similar results in degradability using the *in situ* technique, the gas production technique and the technique of Tilley and Terry. However, this last method is not dynamic and is an endpoint measurement.

4.6. Ranking of alternative techniques

The order of alternative techniques which predicts OMD most accurate (*in situ* technique - gas production technique – technique of Tilley and Terry – pepsin-cellulase technique) as observed by Gosselink et al. (2003b) was similar as the order found in the OMTDR predictions from measured and calculated FOM. This order of accuracy was also similar to the order of techniques that approach the rumen digestion dynamics most closely.

However, this order was found without taking into account the influence of a covariable, such as CP, or a factor, such as method of conservation, on the accuracy of a prediction equation. A prediction equation with a covariable has more chance on a higher RSE than an equation without a covariable, because a second determination in the equation will decrease reproducibility. When comparing OMTDR predictions from measured FOM using one variable or alternative technique, the gas production technique had lowest RSE and highest R^2. Of the alternative techniques predicting OMD used in this study to calculate FOM for OMTDR prediction, only the technique of Tilley and Terry (*in vitro* values) had no covariable.

When the accuracy limit is set the RSE should be lower than 5 % of the mean *in vivo* FOM (600 g $\cdot kg^{-1}$) and only prediction equations with a RSE lower than 30 should be used. Thus OMTDR prediction from the *in situ* technique using DFOM calculation and OMTDR prediction from gp20 separated for fresh and conserved forages could be chosen. Nevertheless OMTDR prediction from the *in situ* technique using DFOM calculation was a validation with a high R^2 (0.80) and OMTDR prediction from gp20 was a regression (R^2 = 0.87).

A disadvantage of the *in situ* technique and the gas production technique, as they approach rumen digestion dynamics most closely, is the high SD of the results and thus the reduced repeatability of these techniques. Thus these alternative techniques

need more repetitions than the more static alternative *in vitro* techniques using enzymes or chemicals.

The *in situ* technique used in this study had probable another disadvantage, because this technique used cows whereas OMTDR was determined in sheep. Caution is needed when extrapolating the results from one species to the other, as these species can differ in ruminal passage rates, feed digestibility (Colucci et al 1990; Dulphy et al., 1994; Poncet et al., 1995) and in degradation characteristics (Šebek and Everts, 1999). But a good relationship between effective degradable DM and OMTDR was observed in this study.

5. Conclusions

Generally concluded, OMTDR predictions from measured as well as FOM using *in vitro* and *in situ* techniques can be used. OMTDR predictions from measured and calculated FOM had similar accuracy, although OMTDR prediction from measured FOM was a regression and needs validation. The most dynamic techniques, the *in situ* technique and the gas production technique, predict OMTDR more accurate than the other *in vitro* techniques. The best OMTDR prediction from calculated FOM was observed when using the *in situ* technique. The best OMTDR prediction from measured FOM was observed when using gp20 separated for fresh and conserved forages.

The choice for an alternative *in situ* and *in vitro* technique will also depend on costs, time, experience, animal welfare and availability of OMTDR data to validate the predictions. Also the additional information delivered by an alternative technique will be important, especially concerning the information on rumen dynamics. The *in situ* technique and the gas production technique provide also rates of degradation or fermentation of OM and the *in situ* technique can also provide degradation rates of other nutrients.

Part III

Alternatives for protein evaluation of forages in ruminants

Chapter 4

Estimation of the duodenal flow of microbial nitrogen in ruminants based on the chemical composition of forages: a literature review

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Estimation of the duodenal flow of microbial nitrogen in ruminants based on the chemical composition of forages: a literature review

Abstract

The objective of this study was to evaluate the estimation of the duodenal flow of microbial nitrogen (N) in ruminants fed forage only, per kilogram of dry matter (DM) intake, which is the yield of microbial protein (YMP). The estimation was based on the chemical composition of forages. A data file of 62 observations was collected from *in vivo* studies on cattle and sheep fed diets with forage only. A statistical analysis of YMP was conducted with neutral detergent fibre (NDF), crude protein (CP), non structural carbohydrates (NSC), group of forage species (legumes or grasses), method of conservation, physical form of presentation, level of DM intake, animal species, methodology and references as parameters. After a stepwise regression, CP was significant and the most important predictor. NSC or the method of conservation had an extra effect on YMP. On the basis of these three parameters the best fit equations were found and the influence of all parameters on YMP were discussed. Using the data file of this study, the prediction of YMP from the PDI-system was also validated. The statistics of the validation of the PDI prediction were similar to the statistics of the equations from this study. In conclusion, the chemical composition of forages, with or without the method of conservation, is a poor indication for the duodenal flow of microbial N ($g \cdot kg^{-1}$DM intake) in ruminants fed diets with forages only.

Keywords : rumen /microbial nitrogen / legumes / grasses / prediction

1. Introduction

The objective of this literature review was to evaluate the protein digestion in ruminants measured by *in vivo* experiments. This evaluation was done as a part of the revision of the feed protein evaluation system in France, PDI (Vérité et al., 1987). The amount of microbial protein synthesised in the rumen is of importance in this system and is on average 64 % of the flow of protein to the duodenum in ruminants consuming forage diets. The quality of microbial protein is quite constant and high because of their amino acid profile (Clark et al., 1992; Merchen and Bourquin, 1994). However microbial protein flowing out of the rumen can vary, depending on factors

like forage species, physiological stage, method of conservation and physical processing of forages (Merchen and Bourquin, 1994).

Microbial protein flow has been predicted by the daily intake of dry matter (DM) or organic matter (OM) (Clark et al., 1992; Sauvant et al., 1995a; Oldick et al., 1999) or, more precisely, based on an index of organic matter fermented in the rumen (FOM), which is used in the French PDI-system (Vérité et al., 1987) and the Dutch DVE/OEB-system (Tamminga et al., 1994). However the intake of DM or OM is a rough predictor, FOM is estimated from OM digested in the total digestive tract and both predictors comprise rumen available nitrogen as well as carbohydrates.

Microbial growth depends on the amount and availability of nitrogen and energy, supplied by the non structural and structural carbohydrates in feed (Clark et al., 1992; Stern et al., 1994). Structural carbohydrates can be represented by neutral detergent fibre (NDF) and has supplemental effects on microbial growth in the rumen (Van Soest et al., 1991). NDF content in feed DM also affects the rate of carbohydrate digestion, which is the major factor controlling the amount of energy available for microbial growth in the rumen (Hoover and Stokes, 1991; Van Soest et al., 1991). A lower NDF content is accompanied by higher concentrations of non structural carbohydrates (NSC) and crude protein (CP). CP favourably improves the efficiency of microbial growth as long as nitrogen is not limiting and protein is not used as a source of energy (Clark et al., 1992; Stern et al., 1994).

When contributions of these different chemical components of forage DM (CP, NDF and NSC) to the synthesis of microbial protein are known, the estimation of the duodenal flow of microbial nitrogen (N) can be made. The importance of NDF in the estimation of the duodenal flow of microbial N has been shown by Oldick et al. (1999), who estimated the daily flow of microbial N to the duodenum on the base of DM intake and NDF content. Because DM intake explains the major part of the daily duodenal flow of microbial protein (Clark et al., 1992; Sauvant et al., 1995a), the prediction of this flow will be more refined when it is estimated per kilogram of DM intake.

The estimation of the duodenal flow of microbial N in ruminants, fed forages only, from the chemical composition of forages and in gram per kg of DM intake is another approach compared to the calculations of the flow of microbial N from the PDI- or DVE/OEB-system (Vérité et al., 1987; Tamminga et al., 1994). The objective of this study was to evaluate this approach and to validate the calculations from the PDI-system, using a database from the literature. Because concentrates or ground forages have a great effect on the duodenal flow of microbial protein (Faichney, 1993; Merchen and Bourquin, 1994), the selected *in vivo* data were from diets containing chopped or long forages only. The duodenal microbial flow per kg of DM intake is called hereafter the yield of microbial protein (YMP).

2. MATERIALS AND METHODS

2.1. Data file generation

A data file containing 62 observations was generated from 34 studies published during the last thirty years (Beever et al., 1977; Berzaghi et al., 1996; Brake et al., 1989; Caton et al., 1993; Charmley and Veira, 1990a+b; Elizalde et al.,1999a+b; Galloway et al., 1992,1993; Goetsch et al.,1990; Hogan and Lindsay, 1979; Holden et al., 1994; Hume and Purser, 1975; Jones and Goetsch, 1987; Jones et al., 1987a+b; Kawas et al., 1990; Krysl et al., 1989, 1991; Lindsay and Hogan, 1972; Makoni et al., 1995; Merchen and Satter, 1983; Muntifering et al., 1985; Narasimhalu et al., 1989; O'Mara et al., 1997; Perez et al., 1996; Peyraud et al., 1997; Stokes et al., 1988a+b; Thompson et al., 1981; Tjandraatmadja et al., 1993; Van Vuuren et al., 1992; Varel and Kreikemeier, 1994). The 62 observations contain 27 observations with legumes (lucerne: 19 and clovers: 8) and 35 observations with grasses (*Lolium perenne*: 14 , *Dactylus glomerata*: 4 and other grasses: 17).

The experiments with sheep and cattle with cannula in the rumen and in the abomasum or in the proximal duodenum and with a clear description of the experimental conditions were selected. All selected publications contain data of the flow of microbial N to the duodenum and the chemical composition of feed DM, at least CP ($g \cdot kg^{-1}$ DM) and NDF ($g \cdot kg^{-1}$ DM). The determination of NDF was done according to the different techniques of Van Soest et al. (Van Soest and Wine, 1967; Goering and Van Soest, 1970; Robertson and Van Soest, 1981; Van Soest et al., 1991) and the determination of CP was done with the Kjeldahl method. Non structural carbohydrate (NSC, $g \cdot kg^{-1}$ DM) was calculated as OM minus CP minus NDF. As a consequence of this calculation, NSC also comprise low concentrations of lipids (Bauchart et al., 1985), which have a small contribution to the energy delivered to microbial digestion (Demeyer and Van Nevel, 1986).

Table I. Description of the data file: numbers of forages, legumes and grasses in each class of parameters: method of conservation, physical form of presentation and animal species.

	Total	Method of conservation			Physical form of presentation		Animal species	
		Fresh	Hay / dried[a]	Silage	Chopped	Long	Sheep	Cattle
	n	n	n	n	n	n	n	n
All forages	62	14	31	17	30	32	27	35
Legumes	27	3	16	8	13	14	22	5
Grasses	35	11	15	9	17	18	5	30

[a] Artificially dried forages

Other parameters, which might have an effect on YMP and which were clearly described in the publications, were also collected for the estimation of YMP in addition to the main chemical components (CP and NDF) in the analyses (Table I and II). The forages were grouped in legumes and grasses and were not represented by the forage species in the analyses because of the low numbers of data for each species. Data on the method of conservation (fresh, hay or artificially dried forage and silage), physical form of presentation (chopped or long), the level of dry matter intake (DMI, g DM·kg^{-1}body weight) and animal species (sheep or cattle) were also collected. The stage of maturity, which is a characteristic of the forages, could not be used in the analyses, since it was not given precisely in the publications. However, the chemical composition of forages are well related to the stage of maturity of the forages (Merchen and Bourquin, 1994).

Table II. Description of the data file: the values of CP content (g·kg^{-1} DM), NDF content (g·kg^{-1} DM), NSC content (g·kg^{-1} DM), DMI (g DM intake·kg^{-1} BW) and the values of the duodenal flow of microbial N, YMP (g·kg^{-1} DM intake) and EMPS (g·kg^{-1} OM apparently digested in the rumen) and the duodenal flow of non ammonia N (NAN, g·kg^{-1} DM intake) in forages, legumes and grasses.

		All Forages	Legumes	Grasses	Difference Legume - grass
CP	Range	50 – 275	131 – 275	50 – 250	
	Mean (SE)	159 (6.8)	190 (8.8)	137 (8.8)	$P < 0.0001$
NDF	Range	298 – 845	298 – 664	331 – 845	
	Mean (SE)	534 (18.1)	458 (16.8)	593 (25.4)	$P < 0.0001$
NSC	Range	23 – 370	105 – 365	23 – 370	
	Mean (SE)	210 (12.3)	249 (10.0)	180 (18.9)	$P < 0.005$
DMI	Range	10.3 – 30.9	10.3 – 30.9	10.3 – 30.3	
	Mean (SE)	20.5 (0.77)	21.3 (1.10)	19.8 (1.06)	NS[a]
YMP	Range	3.4 – 20.8	6.0 – 20.8	3.4 – 18.7	
	Mean (SE)	11.6 (0.52)	13.0 (0.73)	10.4 (0.68)	$P < 0.005$
EMPS	Range	5.4 – 55.9	8.7 – 55.9	5.4 – 50.9	
	Mean (SE)	26.3 (1.35)	30.7 (2.10)	22.8 (1.55)	$P < 0.005$
NAN	Range	8.5 – 34.8	8.5 – 33.9	10.7 – 34.8	
	Mean (SE)	20.7 (0.74)	21.4 (1.02)	20.1 (1.05)	NS[a]

[a] not significant ($P > 0.1$).
CP: crude protein, NDF: neutral detergent fibre, NSC: non structural carbohydrates, DMI: dry matter intake, YMP: yield of microbial protein, EMPS: efficiency of microbial protein synthesis, NAN: non ammonia N.

2.2. Description of the data file

The chemical components (CP, NDF and NSC) well differentiated legumes and grasses (Table II). Although the ranges of these chemical components in the groups of legumes and grasses were wide, the values in the ranges were continuously distributed. However, the analysis of the difference between these two groups of forages might be biased by the parameter animal species, because experiments on legumes were mainly done with sheep and experiments on grasses with cattle (Table I).

On the contrary to the duodenal flow of non ammonia N per kilogram of DM intake (NAN), the duodenal flow of microbial N, expressed as YMP and as EMPS (efficiency of microbial protein synthesis: g duodenal flow of microbial N per kg OM apparently digested in the rumen), was significantly different between legumes and grasses (Table II). The mean values of YMP and EMPS in the data file were lower for grasses than for legumes. The variation in YMP was less large than the variation in EMPS.

Table III. The description of the classes of the factor methodology used in the statistical analyses of the duodenal flow of microbial N (YMP, g·kg^{-1} DM intake) and of non ammonia N (NAN, g·kg^{-1} DM intake).

Classes	Microbial marker	n	Number of markers used for flow measurement		Type of duodenal cannula	n
YMP						
1	Purine in digesta	32	One	+	Simple	21
			One	+	Re-entrant	9
			Two	+	Simple	2
2	DAPA (diaminopimelic acid)	13	One	+	Simple	4
			Two	+	Simple	9
3	^{35}S (sulfur)	10	One	+	Re-entrant	9
			Two	+	Simple	1
4	Amino acid profile, RNA, Cytosine	7	Two	+	Simple	7
NAN						
1	-		Two	+	Simple	19
2	-		One	+	Simple	25
3	-		One	+	Re-entrant	18

For abbreviations, see table II.

2.3. Statistics

GenStat [2000] was used to statistically analyse the data file and to find the best fit equation for the estimation of YMP and NAN from the chemical composition and the other collected parameters. The parameter method of conservation (MC) contained only 2 classes, fresh forages and others, because YMP was significantly different ($P < 0.05$) between fresh forages and other methods of conservation, but no significant differences were found between the other methods of conservation in the range of NDF content of 400 to 550 $g \cdot kg^{-1}$ DM (Mean values for YMP (± SE) were: 15.4 (1.27) for fresh forages (n = 8), 12.0 (0.96) for hay and dried forages (n = 11) and 11.9 (0.93) for silage (n = 12)). NAN was not significantly different for these methods of conservation.

To account for the variation among experiments or studies used in the data file, the parameters methodology and references were included in the analyses. In the analysis of YMP, 4 classes of methodology were composed on the basis of the marker to measure microbial protein and on the basis of the method of measurement of the duodenal flow, with one or two flow markers and with a different type of duodenal cannula (Table III). In the analysis of NAN, 3 classes of methodology were composed on the basis of the measurement of the duodenal flow (Table III). The parameter references (n = 34) represent the 34 studies used in the data file.

At first the RCHECK procedure of GenStat was used to check the normal distribution of the data in the file. The correlation coefficients between the chemical components, the other parameters, YMP, NAN, DM intake per day (DMd) and the duodenal flow of microbial N per day (Mday) were calculated with the CORRELATE procedure.

Candidate equations to estimate YMP were found by using stepwise regression and the FIT procedure. To reduce overparameterisation and multicollinearity in the model, two selections of predictors were done before the regression procedure. At first, the candidate models were composed from the chemical components and their quadratic terms, using the RSELECT procedure. This procedure calculates the Mallow Cp and selects predictors on the base of the residual sum of squares and the number of predictors. Secondly, the other parameters were added individually to the candidate models using the FIT procedure to find out which parameters and interactions could be significant in each candidate model.

$$Y_{ijklmno} = ß_0 + ß_1C_i + ß_2D_j + E_k + ß_3CD_l + ß_4 CE_m + ß_5DE_n + \varepsilon_{ijklmno} \quad (1)$$

$Y_{ijklmno}$ = YMP or NAN; C_i or D_j = chemical components, NDF ($g \cdot kg^{-1}$ DM), CP ($g \cdot kg^{-1}$ DM) or NSC ($g \cdot kg^{-1}$ DM); E_k = one of the parameters (group of forage species, method of conservation, physical form of presentation, animal species, methodology, DMI or references); CD_l, CE_m and DE_n = interactions between chemical components and the added parameter; $ß_{0 \text{ to } 5}$ = regression coefficients; $\varepsilon_{ijklmno}$ = residual errors.

A stepwise regression analysis of YMP and NAN was done using the candidate models with the chemical components, using the parameters, which were significant in model 1, and using the parameters, which had a significant interaction with a chemical component in model 1.

$$Y_{ijklmnopqrs} = ß_0 + ß_1 C_i + ß_2 D_j + E_k + F_l + ß_3 CD_m + ß_4 CE_n + ß_5 DE_o + ß_6 CF_p + ß_7 DF_q + ß_8 EF_r + \varepsilon_{ijklmnopqrs} \quad (2)$$

$Y_{ijklmnopqrs}$ = YMP or NAN; C_i or D_j = chemical components, NDF ($g{\cdot}kg^{-1}$ DM), CP ($g{\cdot}kg^{-1}$ DM) or NSC ($g{\cdot}kg^{-1}$ DM); E_k or F_l = parameters (group of forage species, method of conservation, physical form of presentation, animal species, methodology, DMI or references); CD_m, CE_n, DE_o, CF_p, DF_q, EF_r = interactions between chemical components and parameters; $ß_{0\ to\ 8}$ = regression coefficients; $\varepsilon_{ijklmnopqrs}$ = residual errors.
Overparameterisation was reduced using only two-way interactions. Multicollinearity in the final candidate models was evaluated by calculating the contribution of each variable to the sum of the squares (regression).
Based on these procedures, candidate equations to estimate YMP and NAN were composed. R^2 (determination coefficient) and the probabilities of the equations and
The difference between the observed and predicted (estimated) flows was calculated as the mean square prediction error (MSPE), according to Bibby and Toutenberg (1977):

$$MSPE = 1/n \sum(O-P)^2 \quad (3)$$

O is the observed value and P is the predicted value and n is the number of observations. The square root of MSPE expressed as the percentage of the observed mean is used as a measure of the prediction error. MSPE was decomposed into the error in central tendency (bias), error due to regression (deviation from regression being one) and error due to disturbances (unexplained variation) (Bibby and Toutenberg, 1977).
These statistical parameters were used to find the best fit equations out of the candidate equations. A decreased R^2 and an increased prediction error of the predictions of YMP and NAN could be expected, because of the high number of variation factors and the small number of available data.
Therefore, the best fit equations were also compared according to a method proposed by Mitchell (1997) The essence of this method is that 95 % of the deviations, calculated as predicted minus observed values, are within the envelope of acceptable precision. The limits of this envelope can be defined with reference to the purpose of the model. In this study, SD (standard deviation) of YMP and NAN in the data file were used as limits. Also the limits 1.2 * SD and 1.5 * SD were used, because it is unreasonable to expect the model to perform as well as the *in vivo* data (Mitchell, 1997).

Table IV. Correlation coefficients ($P < 0.05$) between CP ($g \cdot kg^{-1}$ DM), NDF ($g \cdot kg^{-1}$ DM), NSC ($g \cdot kg^{-1}$ DM), DM intake (DMI, g DM intake$\cdot kg^{-1}$ BW), daily DMI (DMd, g DM intake$\cdot d^{-1}$), references (ref), animal species (ani), group of forage species (for), method of conservation (MC), physical form of presentation (pre), methodology (met) and duodenal flow of microbial N, $g \cdot kg^{-1}$ DM intake (YMP) or $g \cdot d^{-1}$ (Mday) and duodenal flow of non ammonia N, $g \cdot kg^{-1}$ DM intake (NAN).

	CP	NDF	NSC	DMI	DMd	ref	ani	for	MC	pre	met	YMP	Mday
CP	x												
NDF	0.78	x											
NSC	0.48	0.91	x										
DMI	0.41	0.53	0.42	x									
DMd	NS[a]	NS[a]	NS[a]	0.27	x								
ref	NS[a]	NS[a]	NS[a]	0.32	0.35	x							
ani	0.46	0.39	0.25	0.14	0.77	NS[a]	x						
for	0.50	0.46	0.33	NS[a]	0.56	NS[a]	0.66	x					
MC	0.50	0.23	0.20	0.47	0.67	0.25	0.38	0.20	x				
pre	NS[a]	NS[a]	NS[a]	NS[a]	NS[a]	NS[a]	NS[a]	NS[a]	0.35	x			
met	0.36	0.41	0.35	0.28	NS[a]	NS[a]	0.19	NS[a]	0.26	NS[a]	x		
YMP	0.50	0.49	0.41	0.29	NS[a]	NS[a]	NS[a]	0.29	0.22	NS[a]	0.25	x	
NAN	0.60	0.49	0.30	0.14	NS[a]	0.29	0.27	NS[a]	NS[a]	NS[a]	0.41	0.51	NS[a]
Mday	NS[a]	NS[a]	NS[a]	0.31	0.92	0.33	0.63	0.41	0.67	NS[a]	NS[a]	0.40	x

[a] no significant ($P > 0.05$); For abbreviations, see table II.

3. RESULTS

The duodenal flow of microbial N per day was correlated with the daily dry matter intake (Table IV). In the statistical analysis of this flow, the parameters, references or methodology, were significant ($P < 0.05$). These parameters were also significant ($P < 0.001$) in the analysis of NAN, which was correlated with CP (Table IV). Because these parameters were not significant in models to predict YMP, the results are focussed on YMP.

YMP was normal distributed and had the highest correlation coefficients with the chemical components, CP, NDF and NSC (Table IV). The candidate models for the estimation of YMP were based on CP or CP^2, with or without NDF, NDF^2, NSC or NSC^2 (Table V). NDF and NSC, which were correlated, could replace each other. NSC would be more supplemental to CP in the prediction of YMP, because the correlation coefficient between CP and NSC was lower than between CP and NDF.

In the candidate models with CP^2 or CP plus CP^2 the parameter, method of conservation, tended to be significant ($P < 0.1$) (Table V). In the candidate models with
CP plus NSC^2 or CP^2 plus NSC^2 the parameter, group of forage species, tended to be significant ($P < 0.1$), although the interactions between the group of forage species and these chemical components were significant ($P < 0.05$) (Table V).

In all candidate models CP or CP^2 were significant after stepwise regression (Table V). Most candidate models could not be used, because the parameters, references or methodology were significant after stepwise regression. These parameters were not significant in the models with CP, CP^2, CP^2 plus MC, CP plus NSC^2 and with CP^2 plus NSC^2. Neither the prediction with CP^2 nor the prediction with CP^2 plus MC or NSC^2 were better than the prediction with only CP (Table VI). In these models, MSPE were for 100% due to the disturbance and the probability of the estimates, MC or NSC^2, tended to be significant ($P < 0.1$).

Nevertheless a model with CP^2 plus MC or NSC^2 tended to predict YMP more precisely than a model with only CP, because these models had a higher percentage of deviations (predicted minus observed values) within the envelope of acceptable precision with limits of 1.5 * SD (Table VII, Figs. 1a and 1b).

CP^2 and MC were almost orthogonal, because the sum of the squares (regression) of the model with CP^2 plus MC was 313, with only CP^2 was 275 and with only MC was 68, as well as regression coefficients of CP^2 were similar between the model with CP^2 plus MC and the model with CP^2. The parameter group of forage species did not improve the model with CP^2 plus NSC^2 because of multicollinearity and interactions with CP^2 or NSC^2.

Table V. Candidate models with chemical components of forages, CP ($g \cdot kg^{-1}$ DM), NDF ($g \cdot kg^{-1}$ DM) or NSC ($g \cdot kg^{-1}$ DM), significant parameters (group of forage species, method of conservation, physical form of presentation, animal species, methodology, references or DMI) to predict the duodenal flow of microbial N (YMP, $g \cdot kg^{-1}$ DM intake), significant interactions between these chemical components and parameters and the results of stepwise regression of the candidate models inclusive of the significant parameters and parameters from significant interactions.

Candidate models	significant parameters	significant interactions ($P < 0.05$)	Result of stepwise regression
CP			CP
CP + NDF		NDF * references	CP + NDF + references + interactions
CP + NSC		NSC * references NSC * animal species	CP + NSC + references + animal species + interactions
CP^2	method of conservation ($P < 0.1$)		CP^2
CP + CP^2	method of conservation ($P < 0.1$)		CP^2
CP^2 + NSC		NSC * references NSC * animal species	CP^2 + NSC + references + animal species + interactions
CP + NSC^2	group of forage species ($P < 0.1$)	NSC^2 * group of forage species	CP + NSC^2 + interactions with group of forage species
CP^2 + NSC^2	group of forage species ($P < 0.1$)	NSC^2 * group of forage species	CP^2 + NSC^2 + interactions with group of forage species
CP^2 + NDF		NDF * references	CP^2 + NDF + references + interactions
CP + NDF^2		NDF^2 * animal species NDF^2 * methodology	CP + NDF^2 + animal species + methodology + interactions
CP^2 + NDF^2			CP^2

For abbreviations, see table II

4. DISCUSSION

4.1. Duodenal flow of microbial protein and chemical components

CP was the most important chemical component in the estimation of YMP. CP expresses the availability of N for the microbes in the rumen and is positively related to YMP and EMPS as long as nitrogen is not limiting and the protein is not used as a source of energy (Kawas et al., 1990; Clark et al., 1992). NSC had an extra effect on YMP, because of the energy supply. An increasing amount of available NSC in the rumen can prevent the use of CP as a source of energy for microbial growth. However, NSC can have a negative influence on the rumen function (Van Soest et al., 1991; Clark et al., 1992). No limiting effect of NSC on YMP was found in this study, which was a consequence of the use of rations with only forages.

NSC could be replaced by NDF in the prediction of YMP. NDF is important for the rumen function and environment, because NDF does not only have a mechanical function, stimulating rumination and forming a mat in the rumen, but also a biochemical function because of the stimulation of salivation and the buffering capacity (Van Soest et al., 1991). NDF had a decreasing effect on YMP, because a low concentration of NDF in dry matter coincides with a high digestibility of forages and high concentrations of NSC and CP in dry matter. Parallel to this, a low concentration of NDF in DM means a high digestion rate of NDF (Sauvant et al., 1995a), which affects the rate of digestion of carbohydrates (Van Soest et al., 1991). NDF content is also an indicator for the maturity of forages and for the difference between legumes and grasses (Merchen and Bourquin, 1994).

4.2. Duodenal flow of microbial protein and other parameters

When MC was included in the model with CP^2, the prediction of YMP was more precise. MC has different effects on the microbial protein synthesis in the rumen. The duodenal flow of microbial protein was higher for fresh forages than for other methods of conservation, which agreed with the observations of Holden et al. (1994) in an experiment with dairy cows fed Orchard grass. The lower values for silage is a consequence of its lower proportions of water-soluble carbohydrates (Demarquilly, 1977). These carbohydrates are energy, which is rapidly available for the microbial growth in the rumen. The lower values for hay and dried forages may be the result of a decreased rate of ruminal degradation of dietary CP, which diminished the availability of N for microbes in the rumen (Merchen and Bourquin, 1994).

A group of forage species tended to have an effect on YMP, but had interactions with CP^2 and NSC^2. The reason for these interactions is that the content of these chemical components as well as YMP differed significantly between legumes and grasses (Table II). Another reason can be a different slope in the effect of CP content or NSC content on YMP between legumes and grasses, because legumes have a

Table VI. Candidate equations ($P < 0.001$) to estimate the duodenal flow of microbial N (YMP, g·kg^{-1} DM intake) composed from candidate models and parameters in table V.

nr.		Equation								R^2	Prediction error (%)
1.		5.33	+	0.0393	*	CP				0.25	30
	P estimate	< 0.05		< 0.05							
2.		8.06	+	0.000125	*	CP^2				0.26	30
	P estimate	< 0.05		< 0.05							
3.		7.80	+	0.000119	*	CP^2	+ 1.89	for fresh forage		0.28	29
							+ 0	for other MC[a]			
	P estimate	< 0.05		< 0.05			< 0.1				
4.		7.04	+	0.000103	*	CP^2	+ 0.000025	*	NSC^2	0.29	29
	P estimate	< 0.05		< 0.05			< 0.1				

[a] MC = method of conservation.
For abbreviations, see table II.

Table VII. Comparison of predictions of the duodenal flow of microbial N (YMP, g·kg^{-1} DM intake): equations of table VI and the calculation from the PDI-system [(FOM * 23.2 microbial N (g·kg^{-1} FOM)) / DM intake (kg·d^{-1})]. Comparison is based on the % of deviations (predicted flows minus observed flows) inside the envelope of acceptable precision with different limits: 4.1 (= SD of observed flows), 4.9 (1.2*SD) and 6 (1.5*SD).

Equation nr.	Prediction	% of deviations inside the envelope of acceptable precision		
		limit = +/- 4.1	limit = +/- 4.9	limit = +/- 6.0
1.	5.33 + 0.0393 * CP	81	84	89
3.	7.80 + 0.000119 * CP^2 + 1.89 for fresh forage			
	+ 0 for other MC[a]	76	85	94
4.	7.04 + 0.000103 * CP^2 + 0.000025 * NSC^2	77	87	92
PDI	calculation from the PDI-system	75	82	86

[a] MC = method of conservation; For abbreviations, see table II; FOM: fermentable organic matter.

lower digestibility of the cell walls than grasses (Merchen and Bourquin, 1994). This difference was not significant in this study because of the small numbers in the data file.

In some models, animal species were significant in the prediction of YMP (Table V). These models were not useful, because references or methodology were also significant. A difference in YMP between cattle and sheep was expected, because they differ in rumen digestion and passage rates (Colucci et al., 1989; Poncet et al., 1995).

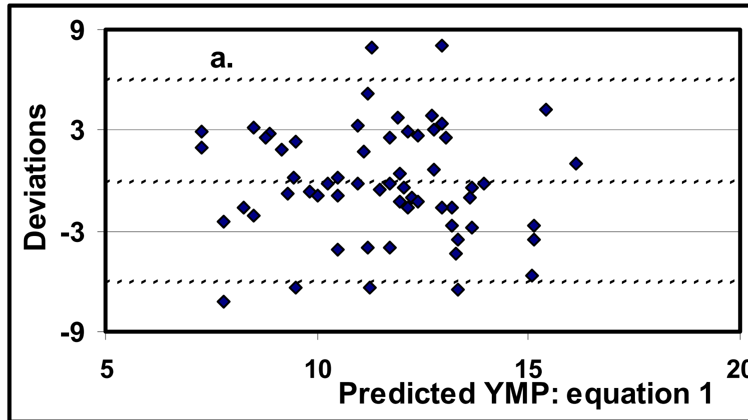
It is noteworthy that the other parameters, which were not significant in the prediction of YMP, may also influence the rates of degradation and passage in the rumen. These parameters, such as the physical form of presentation and DMI, are known to influence microbial protein synthesis. Chopping has a positive effect on DMI through a decreased fill effect and an increased passage rate (Colucci et al., 1990; Djouvinov and Todorov, 1994; Malbert and Baumont, 1989). The efficiency of microbial protein synthesis is positively related to the rumen passage rate as a result of the reducing internal turnover of microbes and reducing maintenance cost for bacterial growth (Van Soest et al., 1991; Walker et al., 1975). The effect of DMI on the passage rate may partly be represented by NDF in the prediction equations, since NDF content is well related to DMI and gastrointestinal fill (Van Soest, 1982). However, the influence of chopping and DMI would have been greater, if the data file did contain diets with ground forages and no restricted DMI (90 % of ad lib).

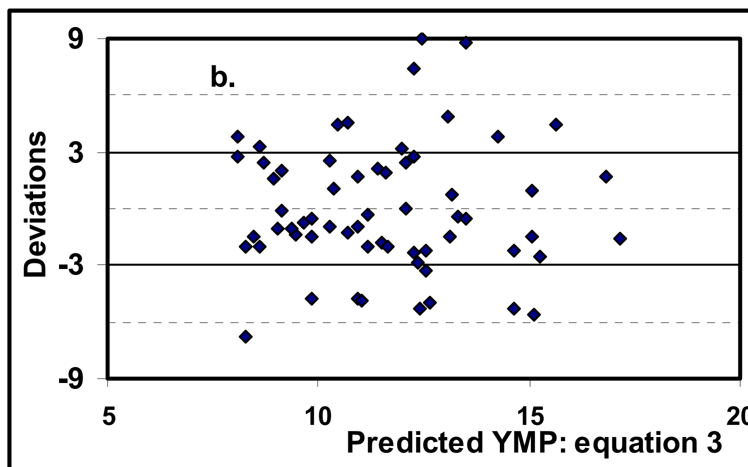
The parameter methodology was significant in some models. The main differences between *in vivo* trials originate from the variation in the methods used for measuring duodenal flow and partitioning protein in microbial versus dietary origin (Faichney, 1993; Stern et al., 1994; Firkins et al., 1998). The parameter references were also significant in some models, due to the heterogeneous origin of the data.

The statistical parameters were poor, the percentages of deviations of the predictions within the envelope of acceptable precision were lower than 95 %, R^2 was low and the prediction error or coefficient of variation (CV) was high. CV was about 30 % and close to the CV (26.3%) of the best fit equation of Oldick et al. (1999). This equation estimates the daily duodenal flow of microbial N from DMI and NDF and is composed on the basis of a data file containing 213 treatments with cattle fed mixed rations.

4.3. Validation of the PDI-system

The statistics of the validation of the calculation from the PDI-system (Vérité et al., 1987) were compared with the statistics of the regressions from this study on the data file of the present study. The PDI calculation was composed using a data file with sheep and cattle and mixed diets and the duodenal flow of microbial N ($g \cdot d^{-1}$)





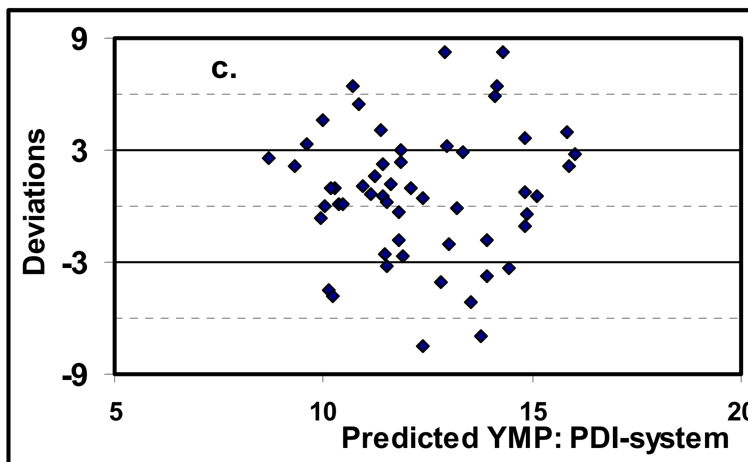


Figure 1 (a,b,c). The deviations (predicted flows minus observed flows) of the predictions of the duodenal flow of microbial N (YMP: $g \cdot kg^{-1}$ DM intake): a. and b., respectively, equation 1 and 3 (Table VII); c. the calculation from the PDI-system [(FOM * 23.2 microbial N ($g \cdot kg^{-1}$ FOM)) / DM intake ($kg \cdot d^{-1}$)]. (------ = limits of envelope of acceptable precision: +/- 6).

was calculated as FOM * 23.2 microbial N ($g \cdot kg^{-1}$FOM). FOM is fermentable OM calculated from OM digested in the total tract (DOM) minus bypass protein, volatile fatty acids and alcohol in silage, and lipids. The values of the PDI calculation were divided with the daily DM intake ($kg \cdot d^{-1}$), to obtain the duodenal flow of microbial N per kg of DM intake. This calculation excludes the great effect of the daily intake of DM or OM on the daily flow of microbial N (Table IV).

When the values of the PDI calculation were related to the YMP values of the data file, R^2 was very low (0.10), the prediction error was 36 % and MSPE was 92 % due to disturbance. The percentage of deviations inside the envelope of acceptable precision (Mitchell, 1997) was also lower than 95 % (Table VII, Fig. 1c). Generally the statistics of the validation of the PDI calculation were similar to the statistics of the regressions from this study.

5. CONCLUSION

The chemical composition of forages, with or without the method of conservation, is a poor indication for the duodenal flow of microbial N per kg DM intake (YMP) in ruminants fed diets with forages only. The precision of the validation of the PDI prediction was close to the precision of the regressions of YMP from this study. The equations from this study need validations with other independent data sets.

Predicting YMP, the yield of microbial protein, is more difficult than the prediction of the daily duodenal flow of microbial protein from DM intake. The prediction of YMP partly implies EMPS, which depends on quantitative, qualitative and dynamic factors of animal and dietary origin. These factors are necessary to improve the predictions of this study and their precision. To integrate all these factors to predict the duodenal flow of microbial N per day or per kg of DM intake, mechanistic rumen models are proposed [Dijkstra et al., 1992; Sauvant et al., 1995b).

Chapter 5

Rumen escape nitrogen from forages in sheep: comparison of *in situ* and *in vitro* techniques using *in vivo* data

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Rumen escape nitrogen from forages in sheep: comparison of *in situ* and *in vitro* techniques using *in vivo* data

Abstract

The objective of this study was to relate *in vivo* data of rumen escape N (REN) of forages with REN estimated from models and with determinations of rumen undegradable N. For these determinations and models measurements from *in situ* and *in vitro* techniques were used. Eleven forages were investigated *in vivo* using sheep with cannula in the rumen, duodenum and ileum. Digesta flows were measured with the double marker technique using ^{51}Cr-EDTA and ^{103}Ru-Phenanthroline. To measure the duodenal flow of microbial N, ^{15}N was infused as well as purin derivatives were measured in urine excretion. *In vivo* REN, expressed as g N $\cdot kg^{-1}$ of N intake or as g N $\cdot kg^{-1}$ of duodenal flow of non ammonia N (NAN), was calculated from duodenal flows of NAN and microbial N and with assumptions for the duodenal flow of endogenous N. REN was also estimated from the models estimating effective undegradable N, using measurements from the *in situ* nylon bag technique or using Cornell Net Carbohydrate and Protein System with data from CPM-Dairy Beta program (CPM-REN). With the *in situ* technique REN was calculated from N residues of forages incubated in the rumen, with and without corrections for microbial contamination. These *in situ* measurements were applied in cows fed a standard diet and in sheep fed the same forage as incubated in the nylon bag. CPM-REN was calculated from five N fractions determined with *in vitro* techniques. Undegradable N of the 11 forages was measured as N residue after 72 h incubation in nylon bags in the rumen of cows (situ residual N), after 24 h incubation with protease and as acid detergent insoluble N (ADIN). REN from different *in situ* measurements and situ residual N had no relationships with *in vivo* data. CPM-REN and the *in vitro* technique using protease had also no relationship with *in vivo* data. ADIN had a moderate relationship with different *in vivo* REN determinations and these relations improved when fresh and conserved forages were separated (R^2 = 0.83 - 0.87; CV = 8 - 16 %). It was concluded, that ADIN has potency to predict *in vivo* REN of forages.

Keywords: nylon bag / ADIN / CNCPS / protease / purine derivatives / ^{15}N

1. Introduction

The duodenal flow of rumen escape nitrogen (N) is an important source of amino acids for ruminants. However the prediction of this flow is rather difficult, as it

depends not only on rumen undegraded protein but also on potentially rumen degradable protein escaping to the duodenum.

Models are used to calculate rumen escape nitrogen (REN) from these two nitrogen fractions measured with different techniques. Firstly the *in situ* technique is used to measure feed N residues after incubation of feed in nylon bags in the rumen and from these residues REN is calculated according to Ørskov and McDonald (1979) or Robinson et al. (1986). Secondly *in vitro* techniques are used to determine N fractions, which are used to calculate REN, as in the Cornell Net Carbohydrate and Protein System (CNCPS) (Sniffen et al., 1992).

In situ and *in vitro* determinations of REN are based on the hypothesis that rumen undegraded N is a measure for REN. These determinations can be N residue in the nylon bag after 72 h incubation in the rumen, after 24 h incubation with protease (Aufrère and Cartailler, 1988) or as acid detergent insoluble N (Van Soest et al., 1991).

The *in situ* method has been the most widely used method and has commonly been used as reference method (Hvelplund and Weisbjerg, 2000), although *in vivo* validations of this method are scarce. When using concentrates, Madsen and Hvelplund (1985) observed a close relationship between *in vivo* and *in situ* measurements for protein degradation. When using forages Vanzant et al (1996) observed no significant difference between *in vivo* and *in situ* measurements, although *in vivo* measurements had large standard errors.

However, to evaluate *in vitro* techniques for predicting REN from forages, the *in situ* technique is not sufficient as long as this method is not well validated. The objective of this study was to relate *in vivo* REN data of forages with REN of forages determined from models and determinations using *in situ* and *in vitro* techniques.

2. Materials and Methods

2.1. Forages

REN of eleven forages was determined in an *in vivo* experiment. These forages (Table 1) were fresh, silage (with formic acid) and hay from Alfalfa (*Medicago sativa*), red clover (*Trifolium pratense*) and orchard grass (*Dactylis glomerata*) and silage (with formic acid) and hay from perennial ryegrasses (*Lolium perenne*). Red clover haylage (a wilted forage wrapped in bales with a dry matter content of about 500 g/kg forage) was made in stead of red clover hay because of wet harvest circumstances.

2.2. *In vivo* measurement

Six wether sheep, fitted with cannula in the rumen, duodenum and ileum, were used (for orchard grass silage five wethers). To calculate *in vivo* REN, duodenal flow of non ammonia N (NAN) and microbial N were measured and duodenal flow of

endogenous N was assumed, as described by Gosselink et al. (2003a). Duodenal digesta flow was measured using a double marker technique (Faichney, 1980) with ^{51}Cr-EDTA and ^{103}Ru-Phenanthroline as flow markers. For microbial N measurements the microbial marker ^{15}N (Gosselink et al., 2003a) and the measurement of the urine excretion of purine derivatives (PD) were used. Therefore also urine was collected to measure the excretion of PD (xanthine, hypoxanthine, uric acid and allantoine), which were analysed with a spectrophotometer. Microbial protein production was calculated from the sum of the derivatives according to Chen and Gomes (1992).

Duodenal flow of endogenous N was assumed to be 1.5 g N $\cdot kg^{-1}$ of DM intake. This value was based on the assumptions used for sheep fed a diet with only forage, as published in the literature: 1.5 gram NAN per day (Siddons et al., 1979), 2.0 gram N $\cdot kg^{-1}$ of DM intake per day (Beever et al., 1987), 2.5 gram NAN per day (Kawas et al., 1990).

Two data sets of *in vivo* REN were used for the comparisons; the first set was based on the use of the microbial marker ^{15}N and the second set was based on PD measurement. *In vivo* REN was expressed as g N $\cdot$ kg^{-1} of N intake, using the markers ^{15}N (N-^{15}N) and PD (N-PD), or as g N $\cdot$ kg^{-1} of duodenal NAN flow, also using the markers ^{15}N (NAN-^{15}N) and PD (NAN-PD).

2.3. *In situ* measurement

The eleven forages were incubated in nylon bags in the rumen of cows fed a standard ration and in sheep fed a diet with only the forage, which is also incubated. The method of sample preparation was described by Dulphy et al. (1999b). Summarised briefly, the samples of fresh forages and silages were lacerated to a particle size of 4-5 mm and put into nylon bags and then quickly frozen in liquid N and preserved at –20 °C. Hays were only ground to a mesh size of 4 mm and put into the nylon bags (Ankom, pore size 30-60 µm, internal surface of 5 * 11 cm and closed by two stitches). The *in situ* measurements were performed according to the procedures described by Michalet-Doreau et al. (1987).

The results from *in situ* measurements of the 11 forages in sheep were taken from Aufrère et al. (2000, 2002, 2003). These measurements had incubation periods of 2, 4, 8, 16, 24 and 48 h and used four sheep. Two replications per sheep were used for 2, 4 and 8 h whereas three replications were used for 16, 24 and 48 h. Using this method the soluble fraction was determined by soaking the bags with the forage in warm water (40 ºC) during 1.5 h followed by drying, in contrast with the *in situ* method in cows which used the soluble fraction calculated by the model (Ørskov and McDonald, 1979).

In this latter method six samples per forage were incubated in the rumen for each incubation period (0, 2, 4, 8, 16, 24, 48 or 72 hours of incubation). Three rumen fistulated cows, fed a ration of hay (70 %) and concentrate (30%), were used and each forage was incubated in duplicate at two different days per cow. After

incubation the bags were kept at –20 ºC until analysis. Prior to the analysis, the bags were thawed and then rinsed with cold water until the water ran clear.

In both methods the bags were beaten for 7 minutes in a "stomacher" (Merry and Mc Allan, 1983; Ould Bah et al., 1988), followed by further washing to remove bacteria and finally dried at 60 ºC for 72 h. DM content of the six residues from the nylon bags was determined and then the residues were pooled per incubation time for N analyses. N residues in the nylon bags were also corrected for residual microbial contamination according to the equation of Michalet-Doreau and Ould-Bah (1989).

Corrected as well as not corrected residues were used to calculate effective degradable N (EDN) using the procedures described in Ørskov and McDonald (1979) and Michalet-Doreau et al. (1987). *In situ* REN was calculated as effective undegraded N, which is 1000 minus EDN ($g\ N \cdot kg^{-1}$ of N intake). The fractional passage rate was assumed to be $6\% \cdot h^{-1}$ as used in the French protein evaluation system (Vérité et al., 1987).

The N residues after 72 h of incubations in the rumen of the cows (*in situ* residual N) were also compared with *in vivo* REN.

Table I. Dry matter (DM, $g \cdot kg^{-1}$) and chemical composition ($g \cdot kg^{-1}$ DM) of the 11 forages.

Forage	method of conservation	Dry matter	Ash	Crude protein	NDF	ADF
Lucerne	fresh	162	138	198	498	346
	silage	212	98	182	438	328
	hay	861	99	171	560	379
Red clover	fresh	127	120	168	492	348
	silage	171	92	166	478	343
	haylage	524	108	128	475	352
Orchard	fresh	193	80	116	676	360
grass	silage	217	71	126	614	343
	hay	852	70	110	697	376
Lolium	silage	191	92	101	578	371
perenne	hay	873	96	91	632	382

2.4. *In vitro* measurement

In vitro techniques were used to measure N fractions. Five N fractions were measured to estimate REN from the model of Cornell Net Carbohydrate and Protein System as described by Sniffen et al. (1992). *In vivo* REN was also related to acid detergent insoluble nitrogen (ADIN) determined as described by Van Soest et al. (1991) and to indigestible N after incubation with protease (protease N: Aufrère and Cartailler, 1988).

The model of Cornell Net Carbohydrate and Protein System is nowadays implemented in the CPM-Dairy Beta program (CPM-Dairy, 2003). To calculate REN from this model (CPM-REN) crude protein was partitioned into five fractions, which were analysed as described by Sniffen et al. (1992) . Fraction A is non-protein N, which is soluble in phosphate-borate buffer (pH = 6.7) and in trichloracetic acid (TCA). Fraction B1 is rapidly degradable true protein and is TCA-precipitated protein from the buffer-soluble fraction. Fraction C is unavailable protein bound to cell walls and is derived from acid detergent insoluble nitrogen (ADIN, % of N total). Fraction B3 is slowly degradable protein and is neutral detergent insoluble nitrogen (NDIN, % of N total) minus ADIN. Fraction B2 is the remaining N and is true protein with an intermediate degradation rate between fraction B1 and B3. Degradation and passage rates were obtained from version 2.0.25a of CPM-Dairy Beta program (CPM-Dairy (2003).

ADIN data were taken from the data measured for CPM-REN (Van Soest et al., 1991).

Indigestible N was measured after 24 h of incubation with proteases in borate/phosphate buffer at pH 8.0 as described by Aufrère and Cartailler (1988) and Cone et al. (1996b). The protease from S. griseus (typeXIV, Sigma P-5147, St Louis, MO, USA) was used in a concentration of 20 mg/l buffer. Tetracyclin (1 mg/l, Sigma N-3503) and nystatin (10 mg/ l, Sigma T-3258) were added to the buffer to prevent microbial growth.

2.5. Chemical analysis

DM contents of feed and residues in nylon bags were determined by drying at 80°C for 48 h and ash content was determined after 6 h at 550 °C. N was determined using the Kjehldahl method (AOAC, 1980), except for N in the residues in the nylon bags, which was determined with the method of Dumas (Merz, 1968). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) (Goering and Van Soest, 1970) were determined on samples dried at 60 °C.

2.6. Statistics

Statistical analyses were done with procedures of Genstat (2002).
The datasets were described with the mean and standard error of the mean (SEM).
To relate REN determined from models and determinations using *in situ* and *in vitro* techniques with *in vivo* REN data, the following model was used:.

(1) *in vivo* REN = β_0 + β_1 * $method_j$ + $factor_l$ + ε_{jkl}

$Method_j$ = REN from the model using the *in situ* technique, CPM-REN, *in situ* residual N, ADIN and protease N; $factor_l$ = forage family (legume or grass), method of conservation (fresh or conserved); β_{0to2} = regression coefficients; ε_{jkl} = residual error, supposed to be normal distributed with zero mean and constant (residual) standard error (RSE). *P* value of the equations and estimates were considered significant when lower than 0.1. Relationships were described with R^2, RSE and coefficient of variation (% CV), which is the ratio of RSE and the mean of the observed data.

Table II. *In vivo* data of 11 forages: DM intake ($g{\cdot}d^{-1}$), the ratio of duodenal NAN flow and N intake (NAN/Nint: $g{\cdot}kg^{-1}$) and rumen escape N ($g\ N\ {\cdot}kg^{-1}$ of N intake or $g\ N\ {\cdot}kg^{-1}$ of duodenal NAN flow) measured *in vivo* using ^{15}N (N-^{15}N, NAN-^{15}N) or purin derivatives (N-PD, NAN-PD)

Forage	method of conservation	DM intake	NAN/Nint	N-^{15}N	NAN-^{15}N	N-PD	NAN-PD
Lucerne	fresh	1528	850	203	239	399	470
	silage	1686	829	238	288	387	467
	hay	1166	891	270	303	423	475
Red clover	fresh	1287	912	114	125	327	359
	silage	1335	910	289	214	500	543
	haylage	1290	1181	380	316	635	528
Orchard	fresh	1332	1089	59	54	379	347
grass	silage	1320	1059	198	187	428	404
	hay	1161	1175	241	205	511	435
Lolium	silage	1271	1374	296	215	576	420
perenne	hay	1300	1429	209	146	576	403
	n	65	65	65	65	65	65
	mean	1334	1069	229	218	469	440
	sem	20.0	25.6	11.3	10.6	14.2	9.2

3. Results

3.1. Forages

The lucerne forages, fresh red clover and red clover silage had a high crude protein (CP) content compared to the other forages (Table I) and their ratio of duodenal NAN flow and N intake was lower than 1000 g NAN · kg^{-1} of N intake (Table II). Red clover haylage had the highest REN and fresh orchard grass had the lowest REN.

3.2. *In vivo* data

In vivo REN values calculated from *in vivo* data using ^{15}N as microbial marker were lower than REN values calculated from *in vivo* data using PD for the measurement of microbial N synthesis in the rumen (Table II). However, these measurements of microbial N ($g \cdot d^{-1}$) using ^{15}N and PD were related: microbial N from PD = 0.69 * microbial N from ^{15}N (n = 11; R^2 = 0.94 ; RSE = 0.67; CV = 4.3 %). The difference between REN per kg N intake (N-^{15}N or N-PD) and REN per kg of duodenal NAN flow (NAN-^{15}N or NAN-PD) was the result of an unstable variable ratio of duodenal NAN flow and N intake (Table II).

The relationships between *in vivo* REN and *in situ* or *in vitro* REN did not improve using the different assumptions for the duodenal flow of endogenous N, as mentioned in the material and methods, to calculate the *in vivo* REN. Other assumptions, 0.181 gram endogenous N per kg $BW^{0.75}$ (Ørskov et al., 1986) or 0.279 gram endogenous N per kg $BW^{0.75}$ (Lintzenich et al., 1995), resulted in similar relationships between *in vivo* REN and *in situ* or *in vitro* REN as when using 1.5 gram endogenous N per kg of DM intake.

3.3. *In situ* technique

Procedures to calculate effective rumen degradable N (EDN) differed slightly between cows and sheep (Table III) and the results obtained with these procedures were poorly related (R^2 was between 0.36 and 0.43). EDN values differed between cows and sheep, although this difference was small when concerning lucerne and large when concerning fresh red clover. The trends in EDN followed the trends in soluble N fraction. The results suggest that the forages had a higher degradable N fraction and degradation rates of N in sheep than in cows. The correction of Michalet-Doreau and Ould-Bah et al. (1989) resulted in a small increase of the degradable N fraction, the degradation rates and EDN.

The four EDN measurements were used to estimate *in situ* REN or effective undegradable N. The values of *in situ* REN were not related with *in vivo* measurements (P > 0.05; Table V). These relationships did not improve when fractional passage rates other than $6\% \cdot h^{-1}$ were used to calculate EDN.
Also *in situ* residual N showed no significant relationships with *in vivo* data (Table V).

Table III. Rumen effective degradable N (EDN: g N ·kg^{-1} of N intake) of the 11 forages of table 1, calculated according to Ørskov & McDonald (1979) with kp at 0.06·h^{-1} and from soluble N fraction (a: g N ·kg^{-1} of N intake), degradable N fraction (b: g N ·kg^{-1} of N intake) and the degradation rate of N (kd, %·h^{-1}), measured in cows (standard diet) or in sheep (same diet as in nylon bag), with (plus) and without (min) the correction of Michalet-Doreau & Ould-Bah (1989).

Forage	Method of	Cow plus				Cow min				Sheep plus				Sheep min			
	conservation	a	b	kd	EDN	a	b	kd	EDN	a	b	kd	EDN	a	b	kd	EDN
Lucerne	fresh	475	416	18.7	789	473	393	18.4	770	412	448	29.8	784	410	421	30.4	761
	silage	665	232	13.1	824	663	206	11.7	799	602	287	21.5	826	601	261	21.4	804
	hay	332	554	10.6	686	328	525	10.0	656	343	542	11.5	693	345	517	10.5	664
Red clover	fresh	664	302	17.2	888	664	284	16.7	873	249	552	35.3	714	249	532	35.5	694
	silage	488	458	17.5	829	488	436	16.9	810	421	446	22.2	763	421	410	22.3	735
	haylage	586	360	8.6	798	585	336	7.4	771	408	496	8.7	698	407	472	7.5	660
Orchard	fresh	328	605	10.0	706	324	576	8.8	666	355	565	10.9	717	355	508	9.9	667
grass	silage	654	282	11.6	840	653	258	8.8	806	527	399	7.9	752	523	370	6.3	705
	hay	378	518	7.7	669	371	489	5.9	613	333	596	4.9	596	324	611	3.5	537
Lolium	silage	694	250	19.3	885	695	208	15.2	844	568	310	20.4	807	566	256	18.8	760
perenne	hay	313	616	11.6	719	313	564	10.0	666	173	729	8.4	597	165	686	7.1	536
	n	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11	11
	mean	507	418	13.2	785	505	389	11.8	752	399	488	16.5	723	397	459	15.8	684
	sem	4.5.	42.9	1.25	23.6	46.2	42.0	1.29	26.2	39.3	38.9	3.01	22.9	39.6	40.2	3.21	25.9

3.4. *In vitro* techniques

CPM-REN was not related with *in vivo* REN (Table IV and V) and poorly related with *in situ* REN (Table VI), measured with cows or sheep on a standard ration and corrected for microbial contamination according to Michalet-Doreau and Ould-Bah (1989). Nevertheless the values from *in situ* REN measured with sheep (sheep-REN) and CPM-REN were close: sheep-REN = 0.997 * CPM-REN ($P < 0.001$; $R^2 = 0.40$; RSE = 5.90 and CV = 21.2 %). CPM-REN had a moderate relationship with indigestible N measured using incubation with protease (Table VI).

Of all determinations ADIN was best related with *in vivo* REN (Table V), but was not related with other determinations (Table VI). The relationship between ADIN and NAN-PD had a lower CV than the relationship between ADIN and N-^{15}N or NAN-^{15}N (Table V). These relationships improved when the method of conservation was included in the regression analysis:

(1)
N-^{15}N = 3.08 * ADIN + 1.6 (fresh) or 15.7 (conserved); $R^2 = 0.87$ and CV = 14 %

(2)
NAN-^{15}N = 3.72 * ADIN + 0.7 (fresh) or 11.6 (conserved); $R^2 = 0.83$ and CV = 16 %

(3)
NAN-PD = 2.74 * ADIN + 29.4 (fresh) or 36.2 (conserved); $R^2 = 0.83$ and CV = 8 %

In these regressions ADIN and method of conservations are orthogonal.

4. Discussion

4.1. *In vivo* data

In vivo REN differed between the calculations from ^{15}N and the calculations from PD, due to their difference of 31 % in measurement of microbial N synthesis in the rumen. This value of 31 % is close to the percentages observed by Perez et al. (1996). This difference between these microbial N measurements can partly be explained by the contamination of endogenous protein with ^{15}N, but mainly be explained by the reference value for microbial N synthesis used to find a relation with the urinary excretion of PD. This reference value was 32 gram microbial N/kg digestible OM fermented in the rumen (Chen and Gomes, 1992) and is 26 % lower than the mean value of 43.2 gram microbial N/kg OM apparently digested in the rumen (n = 11 forages) observed with ^{15}N as microbial marker in the *in vivo* experiment used for this study (Gosselink et al., 2003a). Nevertheless measurements of urine excretion of PD proved useful in many studies on microbial protein production from forage feeding (Tamminga and Chen, 2000).

In this study the comparisons with *in vivo* REN as part of the duodenal NAN flow were also presented as this ratio varied less than the ratio *in vivo* REN as part of N

Table IV. Rumen escape N (g N ·kg^{-1} of N intake) calculated from rumen passage rates (kp: %·h^{-1}), and nitrogen fractions (A, B1, B2, B3, C (ADIN): % of forage N) and their degradation rates (kd: %·h^{-1}), as described in the CNCPS system (CPM-REN), and undegradable N (g N ·kg^{-1} of forage N) measured *in vitro* with proteases (Protease N) and *in situ* as the N residue in nylon bags after 72 h of incubation in the rumen (Situ residual N), using the forages of table 1.

Forage	Method of conservation	CPM-REN	A	B1		B2		B3		C	Kp	Protease N	Situ residual N
				% N	kd	% N	kd	% N	kd	ADIN			
Lucerne	fresh	209	30.5	2.8	200	51.6	15	9.7	2.00	5.4	7.6	398	166
	silage	150	56.8	2.1	150	31.5	11	5.9	1.75	3.7	7.0	234	291
	hay	264	28.0	3.4	150	56.7	9	6.5	1.25	5.4	6.3	463	178
Red clover	fresh	147	42.2	1.2	200	47.8	15	5.3	2.00	3.5	7.6	244	102
	silage	194	40.0	1.8	150	47.4	11	6.6	1.75	4.2	7.0	259	96
	haylage	194	50.6	0.5	150	36.8	9	5.7	1.25	6.4	6.3	181	138
Orchard grass	fresh	248	42.2	6.8	200	31.8	11	17.3	2.00	1.9	6.6	221	100
	silage	210	44.6	2.0	200	40.0	9	11.9	1.75	1.3	5.4	264	141
	hay	275	36.0	2.2	150	43.6	11	16.2	1.50	2.0	5.5	302	168
Lolium perenne	silage	173	45.9	1.4	200	43.2	9	6.5	1.75	3.0	6.1	227	190
	hay	263	17.3	0.6	150	70.9	11	8.9	1.50	2.3	6.4	362	115
	n	11	22	22		22		22		22		22	11
	mean	279	39.5	2.3		45.6		9.1		3.5		287	144
	sem	13.2	2.31	0.42		2.38		0.89		0.35		18.4	7.6

intake, as used commonly. Duodenal NAN flow does not include forage N degraded to ammonia disappearing from the rumen and thus not contributing to escape N and microbial N. When forages had a high CP content, the ratio of duodenal NAN flow and N intake was lower than 100 % (Table I and II), meaning a high ammonia production in the rumen. This ratio was higher than 100 % when forages had a low CP (< 16%) (Table I and II), as a result of the utilisation of N from the urea recycling in the sheep. When escape N or microbial N were expressed as part of duodenal NAN flow, the contributions of these two N sources to the production of amino acids absorbed in the small intestine can be calculated.

4.2. *In situ* technique

Effective degradable N differed between the *in situ* measurements in cows and the measurements in sheep, as a result of different methods of determining soluble N. Determining the soluble N fraction by rinsing and soaking or washing in warm water is more commonly used than the calculation from the model.

The difference in degradation rate of N between the measurements in sheep and cows could be due to a combination of species and ration fed during the incubations. Comparing cows and sheep (Texel ewes), cows displayed 40 % lower degradation rates, whereas dietary roughage : concentrate ratio had no effect (Šebek and Everts, 1999). Cows fed the same forage as incubated with the nylon bags, showed higher degradation rates than cows fed a “standard” brome hay (Vanzant el al., 1996).

Despite all the different *in situ* procedures and calculations, not even a slight relationship between *in situ* and *in vivo* results was found. Compared to *in vivo,* EDN as calculated in protein evaluation systems (Vérité et al., 1987; Tamminga et al., 1994) is less dynamic. These systems used three N fractions, a fixed passage rate and the soluble fraction assumed to be totally degraded. Recently, it was proven that part of the soluble N escapes rumen fermentation and enters the duodenum as amino acids (Aufrère et al., 2000, 2002, 2003).

The *in situ* technique is generally accepted to obtain REN values for feed protein evaluation systems and as reference (Hvelplund and Weisbjerg, 2000). Moreover a tendency towards more confidence in *in situ* measurements than *in vivo* measurements is found in literature. But validations of *in situ* rumen escape N from forages with *in vivo* data are scarce, whereas other recent simple and extensive rumen models already have been validated with *in vivo* data (Bannink et al., 1997; Bateman et al., 2001). As long as both measurements have important limitations, which have often been reviewed (Hvelplund and Weisjberg, 2000; Firkins et al., 1998), this study used *in vivo* data as most reliable reference values.

The most important limitations of *in situ* measurements are its low repeatability and its lack of reproducibility according to Michalet-Doreau and Ould-Bah (1992) and Hvelplund and Weisbjerg (2000), although in this study the same conclusion on repeatability could not be made in the procedures with sheep and cows because of pooled samples per incubation time. However, *in vivo* data can also have high

standard errors. Not much is known about repeatability and reproducibility of *in vitro* methods. Vanzant et al. (1996) observed standard errors from the *in vivo* techniques nearly five times larger than the standard errors from the *in situ* techniques.

Table V. Relations (R^2, RSE, % CV and n = 11) between *in vivo* REN (N-^{15}N, NAN-^{15}N, N-PD, NAN-PD) and REN determined from models using *in situ* measurements in cows and sheep plus correction for microbial contamination (Situ-cow, Situ-sheep) and using *in vitro* measurements (CPM-REN) and the determinations of ADIN, protease N and situ residual N.

REN – determination		N-N^{15}	NAN-N^{15}	N-PD	NAN-PD
Situ-cow	*P*	NS[a]	NS[a]	NS[a]	NS[a]
Situ-sheep	*P*	NS[a]	NS[a]	NS[a]	NS[a]
CPM-REN	*P*	NS[a]	NS[a]	NS[a]	NS[a]
ADIN	*P*	P < 0.1	P < 0.05	NS[a]	P < 0.05
	R^2	0.25	0.46	-	0.47
	RSE	75.9	62.2	-	51.2
	CV	33.0 %	28.5 %	-	11.6 %
Protease N	*P*	NS[a]	NS[a]	NS[a]	NS[a]
Situ residual N	*P*	NS[a]	NS[a]	NS[a]	NS[a]

[a] NS = non significant ($P > 0.1$)

Table VI. Correlations (R^2, $P < 0.05$) between REN determined from models using *in situ* measurements in cows and sheep plus correction for microbial contamination (Situ-cow, Situ-sheep) and using *in vitro* measurements (CPM-REN) and the determinations of ADIN, protease N and situ residual N.

REN – determination	Situ-cow	Situ-sheep	CNCPS	ADIN	Protease
Situ-cow	x	x	x	x	x
Situ-sheep	0.35	x	x	x	x
CPM-REN	0.40	0.40	x	x	x
ADIN	-	-	-	x	x
Protease	-	-	0.58	-	x
Situ residual N	-	-	-	-	-

4.3. *In vitro* techniques

Calsamiglia et al. (2000) reviewed the *in vitro* techniques to predict protein degradation and they concluded that for improving these techniques, further understanding of protein degradation and utilization by rumen microbes and the

ruminant animal is necessary. However, interesting results with forages were observed in this study concerning the determination of REN or undegraded N by *in vitro* techniques.

CPM-REN data were closer to the *in situ* REN data from sheep than from cows, probably due to the determination of the soluble fraction. Compared to the *in situ* technique, the determination of CPM-REN data appeared to be further away from copying the real rumen process. Nevertheless CPM-data will have higher repeatability and reproducibility than *in situ* measurements, because of chemical and *in vitro* determinations of the N fractions and assumed degradation and passage rates. Some authors suggest that Cornell Net Carbohydrate and Protein System predicts REN or N fractions passing to the duodenum better than NRC (1989), which is based on *in situ* measurements (Van Amburgh et al., 1998; Bateman et al.,2001).

Most investigations using proteases from *Streptomyces* griseus are done with concentrates (Calsamiglia et al., 2000). Aufrère et al. (1989) investigated forages and concluded that protein degradation of hay could be predicted with CP content and that protease improved the relationship for heat dried hays. In our study, protease plus CP content had no relationship with *in vivo* REN and a moderate relationship with REN determined from *in situ* measurements ($R^2 < 0.50$).

ADIN can be a simple measurement for predicting REN of forages. ADIN is N associated with lignin as well as indigestible nitrogen (Thomas et al., 1982) or unavailable nitrogen (Van Soest, 1982). ADIN was also related with undegradable N from *in situ* measurement with forages (Vanzant et al., 1996), in contrast to the results in this study. Generally ADIN is used as a measure for heat damage and is used as indigestible or unavailable N in rumen N models (Van Soest, 1982; Sniffen et al., 1992). Modest heating increases ADIN as well as N escaping rumen fermentation and may result in an increased supply of N absorbed from the small intestine (Merchen and Bourquin 1994; Yang et al., 1993). Conserved forages may receive heat during drying, wilting and stocking and consequently their ADIN fraction may show a different relationship with *in vivo* REN compared to fresh forages.

The part of REN, which is potentially rumen degradable N, entering the duodenum is more difficult to explain by ADIN than the part of REN, which is rumen undegradable N. However, ADIN was related with apparent N digestibility (Thomas et al., 1982) and with forage digestibility (Van Soest, 1982). Increasing ADIN decreases digestibility and consequently rumen passage rate, which affects REN.

5. Conclusion

ADIN has potency to predict *in vivo* REN, although this prediction needs validation. The determination of ADIN is cheap, fast and does not impair animal welfare.

Part IV

General Discussion

Introduction

1. Evaluation of alternative techniques for future use

2. Implication of alternative techniques for practical use

General discussion

Introduction

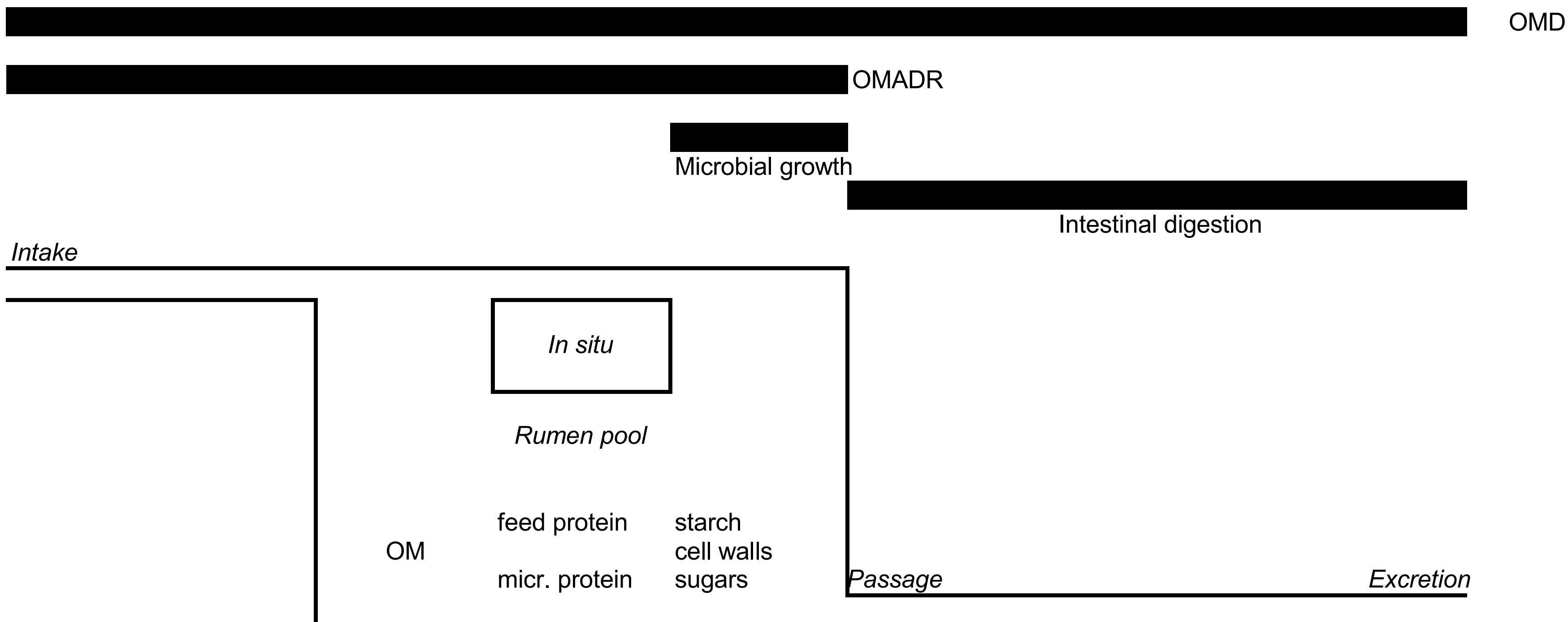
Forages are important feeds for ruminants and the evaluation of their nutritive value is therefore of utmost importance. Among forages a wide variation exists in terms of species, growing conditions, stage of maturity, harvesting and storage conditions. To complicate things further, ruminants posses a complicated digestive systems, the dynamics of which are highly variable but not well understood. As a consequence of microbial fermentation in the forestomach preceding digestion in the intestines, the nature of the postruminal supply of nutrients differs widely from that in the ingested feed. This nature also depends on the compartment of the total digestive tract in which digestion occurs. A schematic representation of the digestive system of ruminants is presented in figure I. Nowadays energy supplied to ruminants, derived from the organic matter ingested, is divided in ketogenic, glucogenic and aminogenic nutrients. An accurate prediction of the supply of these classes of nutrients is an important goal in present feed evaluation research.

This thesis was focussed on the nutritive evaluation of OM and the class of aminogenic or the N containing nutrients, but the prediction of the flow of other classes of nutrients (Figure I) will have similar difficulties as the prediction of nutrient N. The prediction of the rumen digestion and the duodenal flow of nutrients is important for the prediction of the supply of glycogenic, ketogenic and aminogenic precursors, that are necessary for health and production of the ruminant animal. The prediction of OM digested in the total tract (OMD) and truly digested in the rumen (OMTDR) was more successful because they contain digestion or fermentation of all nutrients together in respectively the whole digestive tract and the rumen. It is easier to find alternative techniques, which can mimic the main part of the rumen process than to find techniques mimicking only a part of this process.

As a result of these considerations a division has to be made in the evaluation of the alternative techniques between those for practical routine use in present feed evaluation and those for future use in mechanistic models of the rumen. For practical use, the techniques should mimic the main part of the rumen OM digestion or a part of the rumen digestion of a nutrient, which can be implemented in a simple mathematical rumen model. For future use the techniques should mimic the digestion of an individual nutrient to implement it in a large mechanistic rumen model. Such a model mimics the total rumen function with the integration of the digestion of nutrients and it can be used practically in the future. However, in such models also passage rates must be assumed or predicted from ration characteristics.

The inclusion of degradation rates from *in situ* data and *in vivo* measured passage rates, as measured in chapter 1a and 1b, in the mechanistic rumen model of Dijkstra et al. (1992) using the 11 forages from chapter 5 gave promising results. Bannink et al. (1997) found also good results in validating the model of Dijkstra et al. (1992).

Figure I. Schematic view of the digestive tract



Intake/pool	Turnover = Degradation rate + Passage rate
Passage/pool	Passage rate
Digestion and absorption	Passage - Excretion

(Intake - Excretion)/ Intake	OMD = OM digested in the total digestive tract
(Intake - Passage)/Intake	OMADR = OM apparently digested in the rumen
(Intake - Passage + Micr. protein)/Intake	OMTDR = OM truly digested in the rumen
(Micr. protein*Passage rate)/OMTDR	EMPS = efficiency of microbial protein synthesis

Mathematical integration of new and existing data and concepts into mechanistic models is essential in order to utilize the information optimally and to improve the prediction (López et al. (2000). Degradation data obtained from *in situ* and *in vitro* techniques can be upgraded by using them in a rumen model, which also contains the influence of the ration on the rumen environment. Generally the aim of developing *in situ* and *in vitro* techniques, with the aim to approach the rumen function and environment as much as possible, often compete with claims towards animal welfare, low costs, low demand for labour, low variance and standardisation.

The first objective in this general discussion was to evaluate the alternative techniques studied in this thesis on their possibilities for use in the near future in practical feed evaluation as well as in mechanistic rumen models. The disadvantages of the techniques are discussed in the general introduction. The second objective was to implicate alternative technique with good results in this thesis for practical use in present forage evaluation.

Literature can also be used for generating *in vivo* data to investigate new and existing ideas; examples can be found in Sauvant et al. (1995b) or Oldick et al. (1999). The use of *in vivo* data from literature requires good statistical analyses because of the high variance in the data set as a result of differences in methodology between publications. Consequently a large part of the observed relationships is often unexplained, thus a validation with another independent data set of one experiment is necessary. Observed relationships should also be physiologically explainable.

1. Evaluation of alternative techniques for future use

1.1. *In situ* technique

The *in situ* technique approaches the rumen function very close and from the measurements, degradation and degradation rates are calculated. However, mostly the measurements are done in ruminants fed a standard ration and thus the rumen environment is not adapted to the feed, which is incubated in the nylon bag. A great advantage of this technique compared to the other alternative techniques is that the degradation and degradation rates of the nutrients N, NDF, starch can be measured simultaneously and used in mechanistic models such as that of Dijkstra et al. (1992). The validation of the degradation and degradation rates of nutrients measured *in situ* with *in vivo* data can be complicated, because repeated and difficult rumen content evacuations are necessary (Aitchison, 1985; Robinson et al., 1987). In mechanistic models as well as in a simple models the choice of passage rates are important.

For practical use of the *in situ* measurements, a simple mathematical model using a passage rate (calculation 3 in the general introduction) is developed to predict the endpoint of an effective rumen (un-) degraded nutrient. In the French and Dutch feed

evaluation systems this model is used to estimate rumen undegradable (escape) N with a fixed passage rate and the assumption that the soluble part is totally degraded. This may not always be valid, because Choi et al. (2002) observed with mixed diets that soluble dietary NAN flow entering the omasal canal was between 5 and 10 % of total dietary NAN and Aufrère et al. (2000, 2002, 2003) observed with forage diets similar percentages of soluble dietary NAN escaping rumen degradation.

Chapters 2 and 3 showed that for the prediction of OMD and OMTDR, effective degradable DM calculated with a fixed passage rate of only 3 %/h showed best results, whereas in the present Dutch and French protein evaluation systems, the ruminal passage rates for forages are assumed to be 4.5 %/h and 6 %/h respectively (Vérité et al., 1987; Tamminga et al., 1994). Also a passage rate that varied with the degradation rate of DM showed good results. Passage rates differs between the liquid phase and the particle phase in the rumen, as measured with ^{51}Cr-EDTA and ^{103}Ru-phenanthrolin in chapter 1a and 1b. Such different passage rates can probably be used in mechanistic models, as the model of Dijkstra et al. (1992) is calibrated with such measurements.

Nevertheless the prediction of rumen escape N (REN) using effective undegraded N (chapter 5) did not improve when different passage rates were assumed, probably due to the high soluble N fraction in the forages. When choosing a passage rate to calculate rumen effective degradability of a nutrient with a simple model, the passage rate of 3 %/h, which showed good results in predicting OMTDR, should be taken as the starting point. Thus for nutrients associated with the particle phase, as NDF, a rumen passage rate of about 2.5 to 3 % / h and for nutrients associated with the liquid phase a passage rate of around 8 %/h should be chosen. Though, a change of models using other passage rates or using a coefficient for soluble N escaping rumen degradation, should be accompanied with a validation with *in vivo* values.

1.2. *In vitro techniques*

The development of *in vitro* techniques is important to reduce costs and the use of animals and to improve repeatability and reproducibility. However *in vitro* techniques lack most aspects of the *in vivo* rumen function, as described in the general introduction. The success of an *in vitro* method depends partly on its degree of standardization and partly on the robustness of the reference *in vivo* or *in situ* data base (Tamminga and Williams, 1998).

1.2.1. Gas production technique

For the practical use in present feed evaluation the gas production technique (GPT) can be used for predicting OMTDR and OMD. The advantage of the GPT is that also the rate (ml gas/ g OM / h) of fermentation can be determined. The challenge is to find a method which can attribute GPT kinetics to the degradation of individual carbohydrates, or at least discriminate between non-structural and structural

carbohydrates. As most forages have no starch, the fermentation of sugars can be attributed to the first hours of gas production and after these initial hours the gas production can be attributed to degradation of NDF. When feedstuffs contain starch, it will be more difficult to separate the gas production of the second period between the degradation of starch and the degradation of NDF. Promising is the relationship between starch degradation and gas production observed by Chai et al. (2004), using maize silage samples and starchy feed ingredients.

Starch degradation can be measured from residues after different times of incubation (Chai et al., 2004) and the degradation of NDF can be estimated by measuring the NDF residue after incubation (Blümmel and Becker, 1997). It would be interesting to calculate degradation rates of these nutrients from the residues. When also microbial protein is measured after the same periods of incubations, probably the degradation of carbohydrates at a certain incubation time can be attributed to the synthesis of microbial protein. As a consequence of residues containing microbial protein, the measurement of protein degradation during the fermentation is more difficult. However, branched volatile fatty acids can be estimated in the liquid phase after incubation as indicators of protein fermentation, but more research is necessary.

When the degradation rates of starch and NDF are known or when the gas production rate as a measure for degradation rate is known, a simple model with an assumed passage rate can be used to predict the effective rumen degradable carbohydrate. Mechanistic rumen models can also be developed with these kinetic parameters. Like models using *in situ* data, should also these models should be validated with *in vivo* values. Relationships between GPT parameters and parameters reflecting the dynamics of *in situ* degradation were good for forages (Cone et al., 1998, 1999), but moderate for concentrate feed ingredients (Cone et al., 2002).

The measurement of the synthesis of microbial protein during fermentation in the GPT, as a measure for the duodenal flow of microbial protein, is complicated because in the GPT the passage of microbial protein out of the rumen is not mimicked. *In vivo* ruminal outflow rate has an effect on the yield of microbial protein (gram duodenal flow of microbial N per kg of DM intake) as observed by Gosselink and Poncet (2002; chapter 2). This effect was also observed by Hoover et al. (1982) in a continuous fermenter and with a yield expressed as gram microbial N production per kg of DM digested. Despite the differences between *in vivo* and *in vitro*, the microbial protein synthesis (MNS) in the GPT was measured for better understanding of the GPT. The hypothesis was: when the rate of gas production is maximal, the microbial mass in the gas production technique and the efficiency of MNS (g microbial N / kg OM incubated or fermented) are maximal. This hypothesis should ideally at least result in a ranking of forages similar to the *in vivo* values.

The measurements started by measuring the incubation period, at which the rate of gas production for each forage was maximal (calculation 6 in the general introduction; Groot et al., 1996), using the 12 forages from chapter 3. A second

incubation of the 12 forages was stopped after this incubation period and the amount of purines was determined, using the method by Zinn and Owens (1986), modified by Obispo and Dehority (1999), as a measure of microbial protein. Also the amount of microbial protein in the blank was determined to calculate MNS. The result of this experiment was that MNS was very low compared to the amount of microbial protein in the blank, even when the rumen fluid was more diluted than routinely used (1:9 v/v in stead of 1:2 v/v) and that the repetitions varied a lot. Therefore no reliable relationship was observed between MNS from this experiment and *in vivo* values.

1.2.2. Technique of Tilley and Terry

The *in vitro* technique of Tilley and Terry (1963) uses also buffered rumen fluid to measure OM degradation of feed and is an end point measurement resulting in a low variance. This technique was developed to predict OMD and FOM (fermentable organic matter) calculated from OMD (Vérité et al., 1987; Tamminga et al., 1994). FOM measured with this technique had a good relationship with OMTDR with low residual error. Probably this technique can be used to measure the degradation of carbohydrates, but the measurement of dynamic parameters will be difficult. For these measurements GPT is a better alternative.

1.2.3. Techniques using enzymes and chemical composition

The techniques using enzymes and chemical composition can be done quick with low costs, can be standardised easily and have a high repeatability and reproducibility. These advantages should be considered when comparing the results from these simple techniques with *in vivo* data, because it is obvious that their prospects predicting *in vivo* values are reduced compared with other alternative techniques.

In this study the enzymatic techniques using pepsin-cellulase or protease were used to determine an endpoint measurement of degraded DM and N. For practical use in present feed evaluation, the pepsin-cellulase technique can be used for predicting OMD or OMTDR. For the use of this enzymatic technique in a mechanistic model, a degradation rate should be measured by determining the OM degradation per hour. Probably different carbohydrates can be determined in the residues after incubation with cellulase.

The protease technique as used in chapter 5 is not satisfactory for measuring N degradation, but can be useful in a model predicting ruminal N degradation. In this technique far less N is degraded than in the rumen *in vivo*. This technique can be useful as a model when determining the fast degradable part and the degradation rate, although passage rates should be assumed. When enzymes are used for measurements during many hours, enzymes should be refreshed during incubation of feed.

Probably the protease technique can be used in a model such as the Cornell Net Carbohydrate and Protein System. This mathematical model uses N fractions determined with chemical methods and is still changing as a result of new ideas, as the assumptions for degradation and passage rates have been changed. These rates were changed and adapted between the first development (Sniffen et al, 1994) and the model for practical use nowadays (CPM-Dairy, 2003). The great advantage of rumen models, especially mechanistic model, is that they can be easily adapted to new ideas or research results. However, the models should be validated with *in vivo* data after adaptations, as new regressions also should be validated.

Most feed evaluation systems need models or determinations which can easily predict MNS and REN (respectively microbial and feed protein in figure I). Chapter 4 and 5 showed that the prediction of microbial N fraction using CP is poor and the prediction of REN using ADIN is more promising, but both predictions should be validated as done in the next paragraph 2. Feed values determined by techniques using chemicals are interesting, as their determinations can be done routinely, quickly and standardised.

1.3. Other alternative techniques

For routine and practical use, near infrared reflectance spectroscopy (NIRS) is a routine technique allowing a rapid evaluation of forages regarding appropriate supplementation or forage upgrading (Deaville and Flinn, 2000). NIRS has only value when spectra can be related to reliable *in vivo* values.

Broderick (1987) developed an *in vitro* inhibitor procedure for estimating ruminal protein degradation by using chloramphenicol and hydrazine sulphate to inhibit microbial protein synthesis and amino acid and ammonia N utilisation by ruminal microbes. Results of protein degradation kinetics can be useful in rumen models.

2. Implication of alternative techniques for practical use

A great advantage of the use of *in situ* and *in vitro* techniques compared to the *in vivo* technique is the simplification and the standardisation, allowing their implication in present feed evaluation for ruminants and in future rumen models. In this paragraph good results of some alternative techniques from this thesis are implicated for practical use in forage evaluation.

This paragraph will not discuss further the energy evaluation of forages in ruminants, because good alternative techniques to measure OMD and FOM are developed and discussed in chapter 2 and 3. Energy for the animal can be predicted from OMD (CVB, 2001) and energy for microbial activity in the rumen can be predicted from FOM calculated from OMD (Vérité et al., 1987; Tamminga et al., 1994). FOM measurements from alternative techniques need however proper validation with *in vivo* values before they should be implicated.

Table I. DM content (g/kg) and content of CP (g/kg DM), DM intake (DMI, g/d), FOM (g/kg DM) calculated according to Vérité et al. (1987), ruminal microbial N synthesis measured with the method of Chen and Gomes (Chen, g/kg DMI), soluble (a: g/kg) and degradable (b: g/kg) N fractions and degradation rates (c: /h) measured with the *in situ* technique, and ADIN (% of forage N) of the forages of database 77 (Dulphy et al., 2003a+b and unpublished data from INRA, Clermont-Ferrand-Theix, Unité de Recherches sur les Herbivores, France)[a].

Forage	Method of conservation	DM	CP	DMI	FOM	Chen	*In situ* technique (N)			ADIN
							a	b	c	
Lucerne	fresh	171	227	1797	471	15.0	580	352	0.157	5.3
Lucerne	fresh	162	198	1747	445	11.9	475	416	0.187	8.1
Lucerne	fresh	159	172	1581	427	12.4	538	369	0.215	8.6
Lucerne	fresh	164	199	1906	484	12.9	596	339	0.254	6.5
Lucerne	fresh	107	182	1657	517	15.1	476	483	0.130	23.7
Lucerne	fresh	191	165	1763	484	12.6	489	460	0.151	15.3
Lucerne	fresh	280	129	1935	488	10.1	482	454	0.124	13.0
Lucerne	silage	206	168	1562	367	8.9	778	143	0.152	7.1
Lucerne	silage	212	163	1480	408	10.0	664	232	0.131	6.7
Lucerne	silage	202	183	1805	379	11.1	800	159	0.167	5.6
Lucerne	silage	212	177	2050	394	11.1	675	281	0.154	4.7
Lucerne	silage	213	151	1827	364	9.0	690	246	0135	17.1
Lucerne	silage	216	143	1938	400	9.7	629	306	0.114	17.8
Lucerne	haylage	397	130	1520	427	9.3	700	239	0.140	8.8
Lucerne	haylage	610	141	1404	473	10.1	610	330	0.114	9.2
Lucerne	haylage	700	108	1265	470	8.7	530	397	0.098	13.8
Lucerne	hay	861	165	1190	474	11.6	332	554	0.106	6.5
Lucerne	hay	885	139	1427	426	8.8	328	567	0.114	8.1
Lucerne	hay	858	176	1539	429	9.3	233	683	0.106	6.1
Lucerne	hay	881	94	1488	467	9.2	345	562	0.123	28.7
Red clover	fresh	87	212	1448	556	17.8	561	412	0.228	8.5
Red clover	fresh	127	169	1551	550	13.4	664	302	0.172	16.0
Red clover	fresh	169	159	1704	526	12.3	525	418	0.152	16.4
Red clover	silage	171	157	1452	425	8.6	488	458	0.175	15.0
Red clover	haylage	524	123	1299	498	9.6	585	360	0.086	14.6
Red clover	haylage	469	133	1204	397	8.7	396	522	0.111	12.7
Red clover	haylage	649	98	1248	476	8.3	336	572	0.092	18.2
Red clover	hay	850	108	1233	464	9.8	284	617	0.106	14.8
Ryegrass	fresh	112	171	1175	610	17.3	518	458	0.167	3.5
Ryegrass	fresh	128	129	1198	594	13.7	479	473	0.185	5.2
Ryegrass	fresh	178	87	1058	502	12.0	594	320	0.092	7.6
Ryegrass	fresh	181	92	1204	545	12.4	411	501	0.181	6.0
Ryegrass	fresh	135	194	1354	618	17.2	381	602	0.170	3.6
Ryegrass	fresh	165	112	1142	607	12.7	500	453	0.236	6.1
Ryegrass	fresh	202	83	1112	503	11.9	539	333	0.136	11.0
Ryegrass	fresh	182	86	1363	551	12.1	476	448	0.169	5.0
Ryegrass	fresh	200	100	1847	635	13.4	342	623	0.146	4.4

[a] Table I is continued on the next even page.

The evaluation of the crude protein or N value of forages is more complicated as it is explained in the introduction of this general discussion. However simple predictions of the synthesis of microbial N in the rumen (MNS) and rumen escape N (REN) using chemical components from part III were promising but need validation. They can be determined routinely and they can have supplemental value in the current systems of protein evaluation (Vérité et al., 1987; Tamminga et al., 1994) as an extra indication for these two N fraction. The objective of this paragraph is to validate the best prediction of MNS from chapter 4 and the prediction of REN using ADIN from chapter 5 using the forages from this thesis: database 11 and database 77. These validations are compared with the predictions of the current French protein evaluation system.

Database 11 consists of the 11 forages (chapter 5), which contains also *in vivo* ruminal data measured in chapter 1. Database 77 contains 77 forages (chapter 2) that were selected on the basis of the availability of data (Dulphy et al., 2003a+b; unpublished data from INRA, Centre de Clermont-Ferrand-Theix, Unité de Recherches sur les Herbivores, France) on chemical composition, FOM and effective degradable N measured and calculated according to the French protein evaluation system (Vérité et al., 1987), measurement of MNS according to Chen and Gomes (1992) and ADIN (Table I). For effective degradable N (kp = 0.06 /h) *in situ* measurements in cows were used, as described in chapter 5 and by Dulphy et al. (2003a). Sheep were used to measure OMD (chapter 2) for calculation of FOM and to collect urine for the measurement of MNS according to the method of Chen and Gomes (192), as described in chapter 5 and by Dulphy et al. (2003b).

Firstly an alternative approach of forage N evaluation is described in this paragraph. Secondly the prediction of MNS is evaluated using *in vivo* values measured with ^{15}N as microbial marker and according to the method of Chen and Gomes (1992). Consequently the prediction of REN is evaluated using different predictions of MNS and the alternative approach of forage N evaluation.

2.1. Alternative approach for forage N evaluation

At first the current French and Dutch protein evaluation systems (Vérité et al., 1987; Tamminga et al., 1994) are described briefly. The rumen part of these systems aims at the estimation of the supply of true protein or amino acids from feed to the duodenum. This supply consists of two N (crude protein = 6.25 * N) fractions. The first N fraction is MNS, which is estimated by multiplying FOM intake (energy calculated from OMD) with 23.2 (France) or 24 (the Netherlands) gram microbial N per kg of FOM intake. The second N fraction is REN measured with the *in situ* method and calculated from effective rumen degradable N (Michalet-Doreau et al., 1987; Ørskov and McDonald (1979) and multiplied with the factor 1.11. This factor was found after regression of the duodenal NAN flow with the estimation of MNS and the measurement of REN using a database from the literature. Both protein evaluation systems took endogenous losses resulting from digestion into account. The rumen degradable N balance (Dutch OEB) is the difference between effective

Table I: continued.

Forage	Method of conservation	DM	CP	DMI	FOM	Chen	*In situ* technique (N)			ADIN
							a	b	c	
Ryegrass	silage	157	128	1603	466	12.7	789	177	0.145	6.3
Ryegrass	silage	166	110	1261	447	13.8	741	214	0.123	7.9
Ryegrass	silage	205	87	1252	415	11.1	777	148	0.159	7.4
Ryegrass	silage	200	87	974	426	11.1	758	172	0.171	9.0
Ryegrass	silage	189	121	1320	491	13.0	769	187	0.170	6.8
Ryegrass	silage	194	119	1465	489	14.0	634	322	0.135	6.5
Ryegrass	silage	191	100	1276	406	11.6	694	250	0.193	10.9
Ryegrass	haylage	433	96	1337	497	10.3	725	228	0.124	5.4
Ryegrass	haylage	564	79	1237	522	10.3	586	347	0.101	5.3
Ryegrass	hay	839	98	1192	560	12.0	557	373	0.080	7.9
Ryegrass	hay	869	70	1038	464	10.0	317	572	0.063	10.7
Ryegrass	hay	860	81	1168	520	10.1	320	590	0.079	7.4
Ryegrass	hay	846	97	1146	592	12.1	450	485	0.082	5.2
Ryegrass	hay	875	67	897	498	10.0	313	533	0.067	10.0
Ryegrass	hay	873	84	1313	525	11.3	313	616	0.117	5.3
Orchard grass	fresh	196	155	1616	565	13.3	410	583	0.146	3.9
Orchard grass	fresh	193	117	1415	529	11.8	328	604	0.100	5.0
Orchard grass	fresh	212	89	1330	514	11.4	414	529	0.113	6.9
Orchard grass	fresh	325	127	1843	473	12.0	176	794	0.069	5.7
Orchard grass	silage	225	113	1234	465	10.1	654	282	0.116	6.5
Orchard grass	silage	217	121	1110	473	10.0	618	329	0.083	6.4
Orchard grass	silage	295	133	1340	471	10.4	685	271	0.099	6.2
Orchard grass	silage	287	127	1535	468	10.6	571	386	0.085	7.0
Orchard grass	hay	852	110	1320	482	9.1	378	519	0.077	4.7
Orchard grass	hay	875	80	1175	474	8.8	295	629	0.083	5.8
Orchard grass	hay	846	138	1385	512	8.6	169	798	0.075	6.4
Natural grass[a]	fresh	197	179	1541	553	16.0	483	469	0.150	3.7
Natural grass[a]	fresh	221	125	1357	504	12.9	457	457	0.134	6.2
Natural grass[a]	fresh	196	108	1110	448	11.0	513	377	0.106	5.0
Natural grass[a]	silage	230	116	1152	418	8.9	736	189	0.246	3.0
Natural grass[a]	silage	230	115	1144	479	9.7	747	175	0.215	2.8
Natural grass[a]	silage	248	137	1166	472	8.5	639	312	0.092	9.5
Natural grass[a]	haylage	301	156	980	472	8.9	723	242	0.081	5.3
Natural grass[a]	haylage	617	157	1117	548	8.2	431	534	0.067	7.3
Natural grass[a]	hay	859	119	1248	454	9.8	337	558	0.069	3.1
Natural grass[a]	hay	877	86	1111	422	9.0	257	539	0.061	7.3
Natural grass[a]	hay	860	58	975	496	9.8	336	588	0.092	13.3
Natural grass[a]	hay	881	103	1034	500	9.9	174	733	0.102	6.3
Natural grass[a]	hay	875	110	1160	528	9.8	255	638	0.106	14.3
Natural grass[a]	hay	875	168	1707	483	10.3	126	830	0.048	11.5

[a] Grass from natural grassland.

rumen degradable N and microbial N calculated from FOM and is an indication for a deficiency of N or energy for microbial protein synthesis in the rumen.

The alternative approach is based on predicting different N fractions entering the duodenum from easily determined feed characteristics, earlier discussed by Tamminga (1977). At first the total amount of non ammonia N (NAN) entering the duodenum is estimated from forage N and then this flow is partitioned in fractions of MNS, REN and endogenous N. The duodenal NAN flow can be well predicted from the N content of forages (Ulyatt et al, 1988; chapter 1) and from mixed diets (Tamminga et al., 1979), expressed as gram NAN / gram N intake. The three N fractions should fit into this flow resulting in a validation of the predictions of MNS and REN. The endogenous N fraction was assumed to be 1.5 gram N per kg of DMI, as observed in chapter 5. Compared to the current approach, this alternative approach takes N losses from ammonia leaving the rumen into account and recycling of urea in the animal is integrated in this approach as explained in chapters 1a and 5.

The relationship between forage N content and the duodenal NAN flow observed by Ulyatt et al. (1988) is used to implement the alternative approach: Ulyatt-flow = g duodenal NAN flow / kg N intake = 1430 – 16.9 * N content (g / kg OM); duodenal NAN flow was equal to N intake when forage N content was 25 g N / kg OM. This relationship is validated with the *in vivo* data of database 11. The validation had a reasonable R^2 and a low prediction error (PError), although part of this error is due to bias and regression: *in vivo* = 1.07 * Ulyatt (n = 11, R^2 = 0.74, PError = 11.5 %, MSPE due to bias = 24.1, to regression = 34.3 and to disturbance = 41.6: the statistical parameters are described in chapter 2 or 3).

2.2. Prediction of microbial N synthesis

Two predictions of MNS will be compared, the current estimations of MNS according to the French and Dutch protein evaluation system (Vérité et al., 1987; Tamminga et al., 1994) and one based on the estimations as proposed in chapter 4. The major difference between these estimations is that the current evaluation systems used a more precisely measured energy source (FOM) multiplied with a fixed efficiency of MNS (in France 23.2 and in the Netherlands 24 g microbial N per kg FOM) and that the estimations from chapter 4 used a global measure of energy (dry matter intake, DMI) multiplied with a flexible yield of MNS (g microbial N per kg DMI) predicted from the chemical composition.

At first the MNS estimations are validated and compared using the *in vivo* data from database 11. Secondly the best estimation from chapter 4 and the current French estimation are compared using the MNS values measured according to the method of Chen and Gomes (1992) (Chen-MNS) from database 77.

For the statistical analyses Genstat (2002) was used as well as the statistics according to Bibby and Toutenberg (1977), estimating the prediction error (PError) and the mean square prediction error (MSPE), as described in chapter 2 or 3.

Table II. Microbial protein synthesis (g/d) of 11 forages measured with the method of Chen and Gomes (Chen) or *in vivo* using ^{15}N (N15), estimated from FOM determined as in French and Dutch protein evaluation systems and estimated from the best 3 prediction equations chapter 5 (eq 1, 2 and 3).

Forage	Method of Conservation	Chen	N15	French	Dutch	eq 1	eq 2	eq 3
Lucerne	fresh	20.5	30.4	15.8	16.3	20.0	21.9	18.0
	silage	19.1	26.4	17.5	19.5	21.0	19.8	20.9
	hay	13.3	18.2	11.5	11.9	14.0	13.1	12.4
Red clover	fresh	18.0	25.2	17.8	18.5	15.3	16.8	14.4
	silage	12.7	20.1	13.3	15.4	15.8	14.8	15.4
	haylage	12.9	19.5	15.6	16.7	13.4	12.6	13.9
Orchard	fresh	15.5	23.3	16.0	16.5	13.2	15.0	11.8
grass	silage	15.0	21.2	12.7	14.7	13.6	12.8	12.5
	hay	11.9	17.5	12.6	13.0	11.2	10.7	10.0
Lolium	silage	14.5	20.3	12.6	15.0	11.8	11.4	11.9
perenne	hay	14.2	21.1	15.9	16.5	11.6	11.4	11.2

Table III.The statistical results of the comparison and validation of the estimations from table II using the MNS values measured with the method of Chen and Gomes (Chen) or *in vivo* using ^{15}N (N15) as microbial marker.

Comparisons	R^2		PError (%)	MSPE due to (%)		
	Chen	N15	using Chen	bias	Regression	Disturbance
Chen = 1.025 * eq 1	0.47	0.42	13.0	9.0	24.5	66.5
Chen = 1.026 * eq 2	0.62	0.63	11.2	14.4	37.4	48.2
Chen = 1.076 * eq 3	0.27	0.20	16.6	28.9	19.4	51.7
Chen = 1.034 * French	0.42	0.46	13.8	6.9	2.4	90.7
Chen = 0.960 * Dutch	0.43	0.45	13.8	8.1	2.2	89.7

Table IV.The results of the validation of the French and the alternative (altern) estimations of MNS with the MNS values determined according to the method of Chen and Gomes (Chen) using database 77.

Validations	R^2	PError (%)	MSPE due to (%)		
			bias	Regression	Disturbance
Chen = 0 + 0.99 * French	0.30	16.2	0.0	0.6	99.4
= 1.02 + 0.90 * French	0.30	16.2	0.1	0.0	99.9
Chen = 0+ 0.93 * altern	0.18	17.6	0.7	22.3	77.0
= 4.67 + 0.55 * altern	0.36	15.5	0.1	0.0	99.9

2.2.1. Validation of MNS estimations

The 3 candidate-equations from chapter 4 estimating the yield of microbial N (g microbial N / kg DMI), are: eq 1 = 5.33 + 0.0393 * CP; eq 2 = 7.80 + 0.000119 * CP^2 + 1.89 (fresh forages) or 0 (other methods of conservation); eq 3. = 7.04 + 0.000103 * CP^2 + 0.000025 * NSC^2; CP (crude protein) and NSC (non-structural carbohydrates) are expressed as g / kg DM. To estimate MNS according to the Dutch and French protein evaluation systems, FOM were calculated from OMD measured in the same *in vivo* experiment as MNS was measured. These *in vivo* MNS measurements were performed using the methodology of the *in vivo* experiment in chapter 1 with ^{15}N (N15-MNS) as microbial marker or with Chen-MNS. The results of MNS measurements and estimations can be found in table II.

It can be concluded from Table II, thatChen-MNS values were lower than N15-MNS values but they were well related, as explained in chapter 5. The estimated values from different equations and predictions in table II were closer to the Chen-MNS values than to the N15-MNS.

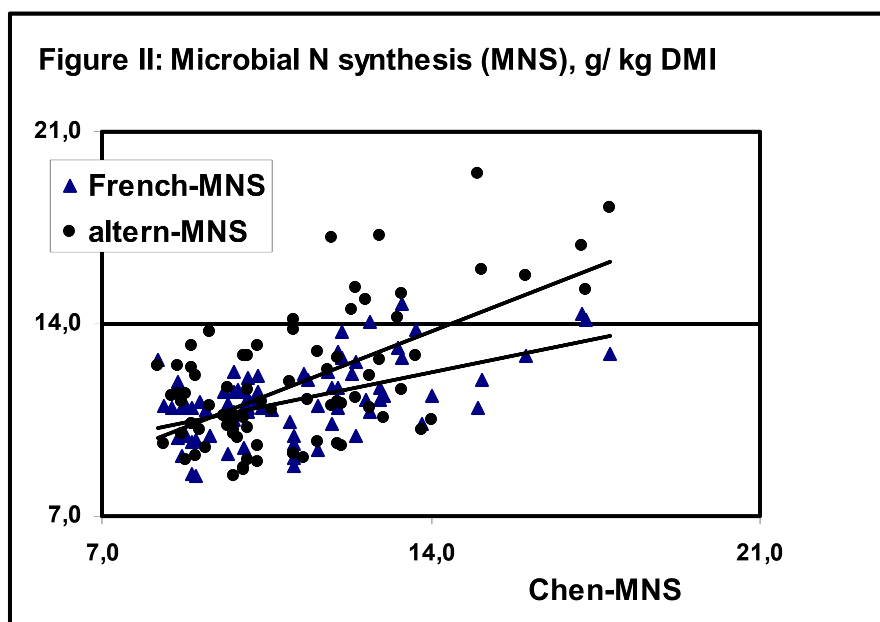
The results of the validation and comparison of the data from table II are shown in table III. The statistics of the validation with Chen-MNS values were similar to the statistics of the validation with N15-MNS. The values of the MNS estimations from chapter 4 and the French and Dutch systems were closer to the Chen-MNS values than to the N15-MNS values. Of all 3 estimations from chapter 4, equation 2 had the best estimation of MNS: highest R^2 and lowest Perror. This prediction of equation 2 had a higher R^2 and a lower PError but a higher percentage of MSPE due to regression than the French and Dutch estimation of MNS. The French and Dutch predictions did not differ in accuracy of MNS prediction.

In conclusion: the prediction equation 2 found in chapter 4 to estimate the yield of microbial N can be an alternative (altern-MNS) for the estimation of MNS of forages according to the current estimations from the French and Dutch evaluation systems.

2.2.2. Comparisons of MNS estimations

In this paragraph the MNS estimations from the French protein evaluation system (French-MNS) and from altern-MNS are compared by validating the values with Chen-MNS values using database 77 (Table IV), the with and without a zero constant. It must be concluded that the French-MNS as well as altern-MNS were poorly related with Chen-MNS and were also not related with each other (Figure II).

From table III and IV can be concluded that the French-MNS and altern-MNS had a similar predictive value. In the following paragraph these two MNS estimations and Chen-MNS together with predictions of REN are compared using the alternative approach of paragraph 2.1.



2.3. Rumen escape N prediction in an alternative approach for forage N evaluation

Two estimations of REN were compared using the alternative approach with the Ulyatt- flow partitioned in 3 fractions, endogenous N, MNS and REN. The 2 estimations of REN were: the current calculation from *in situ* measurements according to the French and Dutch protein evaluation systems (current-REN = 1.11 * effective rumen undegradable N); the estimation from ADIN as it had the best relation with *in vivo* REN in chapter 5 (ADIN-REN = g escape N / kg duodenal NAN flow = 2.74 * ADIN (% of forage N) + 29.4 (fresh forages) or 36.2 (conserved forages).

These two REN estimations were compared in combination with the 3 estimations of MNS from the last paragraph using database 77. Consequently the combination of MNS and REN fitting best in the Ulyatt-flow was chosen, using 1.5 gram N per kg of DMI as the duodenal flow of endogenous N. The combinations are: Chen-MNS plus current-REN (1) and ADIN-REN (2); French-MNS plus current-REN (3) and ADIN-REN (4); Altern-MNS plus current-REN (5) and ADIN-REN (6). Selection criteria were that the calculations of the Ulyatt-flow minus flow of endogenous N minus a combination (Table V) should be close to zero and should have a standard deviation as low as possible. Also the results of conserved or fresh forages and legumes or grasses were calculated separately.

Flows based on the combinations with Chen-MNS (MNS*1.0) were lower than the Ulyatt-flow. When Chen-MNS values are brought at the level of the MNS values measured with the microbial marker ^{15}N from table II (N15-MNS = 1.45 * Chen-MNS), the combination of Chen-MNS values with ADIN-REN was closer to zero than Chen-MNS with current-REN. When arbitrarily Chen-MNS values were multiplied with 1.225 (the middle of 1.0 and 1.45), the combinations with current-REN was closer to

zero. The same results as Chen-MNS can be observed with French-MNS and altern-MNS. Similar results are observed when the database is divided in conserved or fresh forges and legumes or grasses.

The combinations with altern-MNS had 20 to 40 % lower SD than the combinations with Chen-MNS and with French-MNS. The division of the database in conserved or fresh forges and legumes or grasses did not result in lower SD. The combination with the best result, meaning the mean close to zero plus one of the lowest SD, is the combination of altern-MNS at the level of N15-MNS (multiplied with 1.45) and ADIN-REN. The great advantage of this combination is that it is based on CP and ADIN of forages, which can be easily standardised and routinely applied. ADIN had also a good reproducibility, as ADIN of the 11 forages (chapter 5) was determined in Lelystad and ADIN of database 77 was determined at INRA in Theix.

In conclusion: the combination of altern-MNS and ADIN-REN can be used as an alternative or as a first indication for predictions by the current French or Dutch protein evaluation system (Vérité et al., 1987; Tamminga et al., 1994), although probably data on protein requirements should be re-evaluated. Altern-MNS can also be used in the rumen degradable N balance (Dutch OEB) as MNS based on N.

The results of table V suggest also that the French-MNS underestimates real MNS, that the current-REN overestimates real REN and that the combination of French-MNS and current-REN underestimates the total true protein (amino acids) flow to the duodenum. Nevertheless this combination was closer to zero when only the data of grasses were used in the calculations. The explanation of the underestimations is that the French and Dutch protein evaluation systems do not take into account or calculate the ammonia flow to the duodenum and the duodenal flow of endogenous N is assumed differently between both systems and the assumption in this thesis. Another consequence of the underestimation of MNS by French-MNS is the overestimation of the rumen degradable balance.

Conclusions

Alternative *in situ* and *in vitro* techniques can predict simply OMD, FOM and flow of N fractions to the duodenum, what can be useful for practical use in present feed evaluation. Future research on these techniques should be focussed on their contributions to mechanistic rumen models. Mechanistic models should probably replace *in vivo* experiments for producing reference values, because of high costs and reduced animal welfare in *in vivo* experiments. However, these last 2 reasons are also a threat for *in situ* techniques, thus the research on *in vitro* techniques should have priority. Replacing of *in vivo* experiments will require an extensive validation on independent and large datasets, covering a wide range of diets.

Table V. The statistical results (n = 77) of the comparison of the 6 combinations of the estimations of MNS (Chen-MNS, French-MNS, altern-MNS: g MNS / kg DMI) and REN (current-REN, ADIN-REN: g REN / kg DMI) fitting within Ulyatt-flow[1)] using 1.5 g N per kg DMI for duodenal flow of endogenous N.

Combinations		Ulyatt-flow minus combination and endogenous flow	
		mean ± SD (MNS*1.0)	mean ± SD (MNS*1.45)
1. Chen-MNS + current-REN		2.7 ± 3.25	-2.3 ± 3.66
	C [2)]	3.3 ± 3.56	-1.3 ± 3.77
	F [2)]	1.5 ± 2.25	-4.4 ± 2.47
	L [2)]	4.4 ± 3.28	0.4 ± 3.70
	G [2)]	1.5 ± 3.07	-3.6 ± 3.42
2. Chen-MNS + ADIN-REN		6.3 ± 3.26	1.3 ± 3.74
	C [2)]	6.8 ± 3.54	2.3 ± 3.88
	F [2)]	5.2 ± 2.44	-0.7 ± 2.62
	L [2)]	8.4 ± 2.82	3.7 ± 3.32
	G [2)]	5.1 ± 3.17	0.0 ± 3.62
4. French-MNS + current-REN		2.6 ± 3.72	-2.5 ± 4.05
	C [2)]	2.6 ± 4.10	-2.2 ± 4.46
	F [2)]	2.6 ± 3.01	-2.9 ± 3.22
	L [2)]	4.2 ± 3.83	**-0.6 ± 4.13**
	G [2)]	**1.1 ± 3.35**	-4.1 ± 3.54
5. French-MNS + ADIN-REN		6.2 ± 3.54	1.2 ± 3.85
	C [2)]	6.2 ± 3.66	1.3 ± 3.99
	F [2)]	6.3 ± 3.42	0.8 ± 3.69
	L [2)]	8.3 ± 3.26	3.5 ± 3.55
	G [2)]	4.7 ± 3.08	-0.5 ± 3.24
7. altern-MNS + current-REN		2.0 ± 2.58	-3.3 ± 2.93
	C [2)]	2.6 ± 2.75	-2.2 ± 2.64
	F [2)]	0.8 ± 1.74	-5.6 ± 2.13
	L [2)]	2.1 ± 2.80	-3.7 ± 3.27
	G [2)]	1.8 ± 2.74	-3.1 ± 2.89
8. altern-MNS + ADIN-REN		5.6± 1.84	0.3 ± 2.05
	C [2)]	6.2 ± 1.80	1.4 ± 1.32
	F [2)]	4.5 ± 1.36	-1.8 ± 1.51
	L [2)]	6.2 ± 1.89	0.4 ± 2.44
	G [2)]	5.4 ± 1.94	0.5 ± 1.83

1) Ulyatt-flow: n=77, mean ± SD = 20.7 ± 3.6 g duodenal NAN flow / kg DMI.
2) C = conserved forages (n= 51); F = fresh forages (n= 26); L = legumes (n= 29); G = grasses (n= 48)

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Summary

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Résumé

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Samenvatting

Summary

Part I: General introduction

In vivo experiments using cannulated ruminants are laborious and expensive and impair animals. Therefore the past 40 years *in situ* and *in vitro* techniques were developed for feed evaluation, although their validations are scarce. The first objective of this thesis was to validate and to compare these alternative techniques with data from *in vivo* experiments. To investigate the synthesis of microbial N in the rumen a literature study was performed. The second objective was to evaluate and to implicate the alternative techniques for future and practical use in feed evaluation. Techniques were compared concerning the ruminal digestion of single forages and energy values: *in vivo* total tract organic matter digestibility (OMD) and rumen fermentable organic matter (FOM), and protein values: microbial nitrogen synthesised in the rumen (MNS) and rumen escape N (REN).

The second part of the general introduction of this thesis (chapter 1a and 1b) described the experiment measuring *in vivo* data used in this study. These data were the reference values for comparing and validating the alternative techniques. For this experiment sheep with cannula in the rumen, duodenum and ileum, were used to study the digestion of 6 legumes and 6 grasses (database 12). Fresh and ensiled forages, hay of perennial ryegrass, orchard grass and lucerne and red clover haylage were fed to the sheep. Digesta flows and rumen outflow rates were measured using ^{51}Cr-EDTA, ^{103}Ru-Ph and ^{15}N as markers. Chapter 1b also contains original data of 98 forages (database 98) used for the prediction of OMD in chapter 2.

Part II: Alternatives for energy evaluation of forages in ruminants

In this part the *in situ* nylon bag technique and the *in vitro* techniques: the pepsin-cellulase technique, the gas production technique and the Tilley and Terry technique were compared and validated in their prediction of OMD (chapter 2) and FOM (chapter 3). To predict OMD, database 12 and database 98 were used. Database 98 was divided in a database with 37 forages (database 37) with similar qualities as database 12 and a database with 61 forages (database 61). Database 37 was used to find relationships between OMD and the alternative methods. Databases 12 and 61 were used to validate the relationships. Database 61 was also used to find out if dry matter intake had an effect on these relationships. Although there was a ranking in predicting OMD, all alternative techniques showed good potency to predict OMD.

To predict FOM, OM truly digested in the rumen (OMTDR) was regarded as *in vivo* FOM using database 12 (chapter 3). OMTDR was related to FOM measured with *in situ* and *in vitro* techniques and to FOM calculated from OMD measured *in vivo* or predicted from the best relationships of chapter 2. The dynamic *in situ* and gas production techniques gave most accurate OMTDR predictions.

Part III: Alternatives for protein evaluation of forages in ruminants

MNS was evaluated using *in vivo* data from the literature (chapter 4) and several determinations of REN using *in situ* and *in vitro* techniques were compared (chapter 5). Chapter 4 showed a significant relationship between crude protein and MNS. Non-structural carbohydrates and the method of conservation had an extra effect on MNS. From these three parameters the best fit equations estimating MNS were found, although the relationships were poor.

In chapter 5 *in vivo* REN data were related with REN estimated from models and determinations of indigestible N. The models used *in situ* data or *in vitro* data as done in the Cornell Net Carbohydrate and Protein System. Indigestible N was measured with the *in situ* technique, with the protease technique and as acid detergent insoluble N (ADIN). Only ADIN was related with *in vivo* REN. These relationships improved when fresh and conserved forages were separated.

Part IV: General discussion

In the general discussion the alternative techniques were evaluated and implicated for future and practical use in forage evaluation. Their contributions to the prediction of nutrient flows from the rumen using models were discussed. The *in situ* technique gave good results in the different chapters and is currently most suitable to measure rumen degradation and degradation rates of nutrients. The gas production technique (GPT) gave good results in predicting OMD as well as OMTDR. The GPT produces also dynamic aspects of fermentation, although these aspects need more research. An attempt to estimate MNS with the GPT was disappointing. The technique of Tilley and Terry is an endpoint measurement and gave a good OMD prediction.

In vitro techniques using enzymes and chemical characterization could also be of use in rumen models. The pepsin-cellulase technique predicted OMD quite well, ADIN showed a promising relationship with REN and MNS had a promising relationship with chemical composition. These latter two relationships were implicated in an alternative approach of forage protein evaluation.

From the general discussion it was concluded that alternative techniques can be useful in forage evaluation for practical as well as futur use.

Résumé

Partie I: Introduction générale

Les expérimentations in vivo qui ont recours à des animaux ruminants fistulés sont coûteuses en temps et en argent et peuvent faire souffrir les animaux. C'est pourquoi durant les 40 dernières années des techniques *in situ* et *in vitro* ont été développées pour évaluer les aliments des ruminants, bien que leur validation ait été rarement faite. Le premier objectif de cette thèse a été de valider et de comparer les résultats de ces techniques alternatives avec des données provenant d'expérimentations *in vivo*. Pour avoir une bonne idée des résultats de la synthèse microbienne dans le rumen une recherche bibliographique a d'abord été effectuée. Le second objectif a été de mettre en place et d'évaluer des techniques alternatives pour une utilisation future et facile en vue d'évaluer la valeur des fourrages. Ces techniques ont été comparées pour ce qui concerne la digestion ruminale et de la valeur énergétique de fourrages distribués seuls: digestibilité *in vivo* de la matière organique dans l'ensemble du tube digestif (DMO) et matière organique fermentescible dans le rumen (MOF), ainsi que des valeurs azotées : azote microbien synthètisé dans le rumen (AMS) et azote alimentaire échappant à la dégradation ruminale (AAR).

La seconde partie de l'introduction générale de cette thèse (chapitre 1a et 1b) décrit l'expérimentation dans laquelle ont été mesurées les données *in vivo* utilisées dans la thèse. Ces données ont ensuite servi de référence pour comparer et valider les techniques alternatives étudiées. Pour cette expérimentation des moutons, équipés de cannules du rumen, du duodénum et de l'iléon , ont été utilisés pour étudier la digestion de 6 fourrages de légumineuses et de 6 fourrages de graminées (base de 12 données). Des fourrages frais et ensilés, du foin de ray-grass anglais, de dactyle, de lucerne et de trèfle violet, ainsi que du fourrage préfané de trèfle violet ont été distribués aux moutons. Les flux digestifs et les vitesses de sortie du rumen ont été mesurés avec le ^{51}Cr-EDTA, le ^{103}Ru-Ph et ^{15}N comme marqueurs. Le chapitre 1b contient aussi les résultats originaux obtenus pour 98 fourrages (base de 98 données) utilisés pour la prévision du DMO dans le chapitre 2.

Partie II: Techniques alternatives pour évaluer la valeur énergétique des fourrages des ruminants

Dans cette partie la technique des sachets *in situ* et les techniques *in vitro* (pepsine-cellulase, production de gaz, Tilley et Terry) ont été comparées et validées pour prévoir les valeurs DMO (chapitre2) et MOF (chapitre 3). Pour prévoir les valeurs DMO les bases de 12 et de 98 données ont été utilisées. La base de 98 données a été divisée en une base de 37 fourrages (base de 37 données) ayant des caractéristiques similaires à ceux de la base de 12 données, et une base de 61 fourrages (base de 61 données). La base de 37 données a été utilisée pour calculer

les relations entre les valeurs DMO et les résultats donnés par les méthodes alternatives. Les bases de 12 et de 61 données ont été ensuite utilisées pour valider les relations trouvées. La base de 61 données a aussi été utilisée pour rechercher un éventuel effet des quantités ingérées sur les relations obtenues. Bien qu'il y ait une certaine hiérarchie entre les techniques pour prévoir les valeurs DMO, toutes les méthodes alternatives étudiées ont montré une bonne aptitude à prévoir ces valeurs DMO.

Pour prédire les valeurs MOF, les quantités de Matière Organique réellement digérée dans le rumen (MODR) ont été considérées comme représentatives de ces valeurs MOF, en utilisant la base de 12 données (chapitre 3). Les valeurs MODR ont été corrélées avec les valeurs MOF mesurées grâce aux techniques *in situ* et *in vitro* et aux valeurs MOF calculées à partir des valeurs DMO mesurées *in vivo* ou prédites à partir des meilleures relations du chapitre 2. Les résultats de la digestion en sachets et de la production de gaz *in vitro* ont permis de calculer les meilleures équations de prévision des valeurs MODR.

Partie III: Techniques alternatives pour évaluer la valeur azotée des fourrages des ruminants

Les valeurs AMS ont été évaluées à partir des données *in vivo* de la bibliographie (chapitre 4), puis des déterminations des valeurs AAR obtenues à partir de techniques *in situ* et *in vitro* ont été comparées (chapitre 5). Dans le chapitre 4 est donné une relation significative entre les teneurs en Matières Azotées Totales et les valeurs AMS. La teneur en glucides non pariétaux et la méthode de conservation du fourrage ont un effet notable sur ces valeurs AMS. Les meilleures relations à partir de ces 3 paramètres ont été calculées, bien qu'elles ne soient pas très étroites.

Dans le chapitre 5 les valeurs *in vivo* de AAR ont été corrélées avec les valeurs de AAR estimées à partir de modèles et de déterminations de l'azote indigestible. Ces modèles utilisent des données *in situ* ou *in vitro* comme le propose le Système "glucides nets et protéines" de Cornell. Les teneurs en azote indigestible ont été mesurées avec la technique *in situ*, avec la technique pepsine-cellulase, ainsi qu'avec les teneurs en N insoluble de l'ADF (ADIN). Seules les valeurs ADIN ont été bien corrélées avec les valeurs AAR obtenues *in vivo*. Les relations trouvées sont améliorées lorsque les fourrages frais et conservés sont considérés séparément.

Partie IV : Discussion générale

Dans la discussion générale les techniques alternatives ont été évaluées en fonction de leur éventuelle et future utilisation dans l'estimation de la valeur des fourrages. Leur contribution à la prévision des flux de nutriments quittant le rumen à partir de modèles a été discutée. La méthode des sachets *in situ* donne de bons résultats tout au long des différents chapitres et est la mieux adaptée pour mesurer les différentes dégradations dans le rumen et leur vitesse. La technique à partir des

productions de gaz (GPT) donne de bons résultats pour prévoir la digestibilité de la MO ainsi que les valeurs MORD. La technique avec production de gaz apporte aussi des résultats quant aux aspects dynamiques de la digestion, bien que des recherches complémentaires soient encore nécessaires. Une tentative pour prévoir les valeurs AMN avec cette technique n'a pas aboutie. La technique de Tilley et Terry est une mesure de digestion finale, mais elle permet une bonne prévision des valeurs DMO.

Les résultats des techniques *in vitro* utilisant des enzymes et une caractérisation chimique peuvent aussi servir pour modèliser le fonctionnement du rumen. La technique à la pepsine-cellulase permet une bonne prévision des valeurs DMO, les valeurs ADIN offrent des relations prometteuses avec les valeurs AAR et la composition chimique semble un bon prédicteur des valeurs AMS. Ces deux dernières liaisons sont impliquées dans une approche alternative de l'évaluation de la valeur azotée.

A partir de la discussion générale il a été conclu que les techniques alternatives étudiées peuvent être tout à fait utiles dans le futur pour prévoir de façon relativement pratique la valeur des fourrages.

Samenvatting

Deel I: Algemene introductie

In vivo experimenten bij herkauwers met cannules in het maagdarmkanaal kosten veel tijd en geld en verminderen het dierwelzijn. Daarom werden de laatste 40 jaar *in-situ-* en *in-vitro*-technieken ontwikkeld voor voederwaardering. Deze technieken zijn echter nog te weinig gevalideerd. Het eerste doel van dit proefschrift was om deze alternatieve technieken te valideren en te vergelijken met gegevens van *in vivo* experimenten. De synthese van microbieel eiwit in de pens werd onderzocht door middel van een literatuurstudie. Het tweede doel van het onderzoek was om de alternatieve technieken te valideren en te implementeren voor praktisch en toekomstig gebruik in de voerderwaardering. De technieken werden vergeleken op basis van de pensvertering van ruwvoeders en de energie- en eiwitwaarden. De onderzochte energiewaarden waren *in vivo* fecale verteerbaarheid van organische stof (VOS) en pensfermenteerbare organische stof (FOS). De onderzochte eiwitwaarden waren de synthese of microbieel eiwit in de pens (MES) en pensbestendig eiwit (PBE).

Het tweede deel van de algemene introductie van dit proefschrift (hoofdstuk 1a en 1b) beschrijft experimenten om de *in vivo* gegevens te verkrijgen ten behoeve van dit onderzoek. Deze gegevens werden gebruikt als de referentiewaarden om de alternatieve technieken te vergelijken en te valideren. Voor dit experiment werden schapen met cannules in de pens, duodenum en ileum gebruikt om de vertering van 6 vlinderbloemigen en 6 grassen (database 12) te onderzoeken. Lucerne, rode klaver, kropaar en engels raaigras werden in verse vorm, als silage en als hooi gevoerd aan de schapen. Darmdoorstromingen en passagesnelheden in de pens werden gemeten met radio-actieve merkers (^{51}Cr-EDTA en ^{103}Ru-Ph) en ^{15}N. Hoofdstuk 1b bevat ook de originele gegevens voor de voorspelling van VOS van 98 ruwvoerders (database 98).

Deel II: Alternatieven voor energiewaardering van ruwvoeders bij herkauwers

In dit deel zijn de *in-situ*-techniek en de *in-vitro*-technieken (pepsine-cellulase, gas-productie-techniek en Tilley-en-Terry-techniek) vergeleken en gevalideerd op basis van hun voorspelling van VOS (hoofdstuk 2) en FOS (hoofdstuk 3). Hiervoor werden databases 12 en 98 gebruikt. Database 98 werd verdeeld in een database met 37 ruwvoeders (database 37) met vergelijkbare kwaliteit als database 12 en een database met 61 ruwvoeders (database 61). Database 37 werd gebruikt om relaties tussen VOS en de alternatieve technieken te vinden. Databases 12 en 61 werden gebruikt om deze relaties te valideren. Database 61 werd ook gebruikt om een effect van drogestofopname op deze relaties te onderzoeken. Alle alternatieve technieken lieten een hoge potentie om VOS te voorspellen zien.

In hoofdstuk 3 werd met behulp van database 12 *in vivo* gemeten FOS (VFOS) vergeleken met FOS gemeten met *in-situ-* en *in-vitro*-technieken. Eveneens werd een vergelijking gemaakt met FOS berekend vanuit VOS gemeten *in vivo* of vanuit VOS voorspeld met de beste relaties van hoofdstuk 2. De dynamische *in-situ-* en gas-productie-technieken lieten de meest accurate VFOS voorspellingen zien.

Deel III: Alternatieven voor eiwitwaardering van ruwvoeders bij herkauwers

De synthese van microbieel eiwit in de pens (MES) werd geëvalueerd met gebruik van *in vivo* gegevens uit de literatuur (hoofdstuk 4). Er werd een significante relatie gevonden tussen het gehalte aan ruw eiwit en MES en bovendien hadden de niet-NDF-koolhydraten en de conservering van ruwvoeders een extra effect. Met deze parameters werden vergelijkingen gevonden om MES te berekenen, ofschoon ze statistisch zwak waren.

In hoofdstuk 5 werden *in-situ-* en *in-vitro*-technieken gebruikt voor de voorspelling van pensbestendig eiwit (PBE) gemeten *in vivo*. Gegevens van deze technieken werden gebruikt voor modellen die PBE voorspellen zoals in het Cornell Net Carbohydrate and Protein System. Tevens werd onverteerbaar N bepaald met de *in-situ*-techniek, met de protease-techniek en als acid detergent insoluble N (ADIN). Alleen ADIN was gerelateerd aan *in vivo* PBE en deze relatie verbeterde wanneer verse en geconserveerde ruwvoeders apart werden beschouwd.

Deel IV: Algemene discussie

In de algemene discussie werden de alternatieve technieken geëvalueerd en geïmplementeerd voor toekomstig en praktisch gebruik in ruwvoederwaardering. Hun bijdragen aan de voorspelling van nutriëntenstromen vanuit de pens werden bediscussieerd. De *in-situ*-techniek gaf goede resultaten in de verschillende hoofdstukken en is momenteel het meest geschikt voor het meten van de afbraak en de afbraaksnelheid van nutriënten in de pens. De gas-productie-techniek (GPT) gaf goede resultaten in de voorspelling van VOS en FOS. GPT produceert ook dynamische aspecten van de pensfermentatie en meer onderzoek is nodig alvorens ze te kunnen inbouwen in voederwaarderingssystemen. De poging om MES te voorspellen met GPT gaf teleurstellende resultaten. De techniek volgens Tilley en Terry is een eindpuntmeting met een goede voorspelling van VOS.

In-vitro-technieken die enzymen gebruiken en chemische karakteristieken bepalen, kunnen ook gebruikt worden in pensmodellen. De pepsin-cellulase-techniek voorspelt VOS redelijk goed en er waren veelbelovende relaties tussen PBE en ADIN en tussen MES en chemische karateristieken. Deze relaties werden geïmplementeerd en gevalideerd in een alternatieve benadering van eiwitwaardering voor ruwvoeders. De conclusie van de algemene discussie was dat de alternatieve technieken nuttig kunnen zijn bij de ruwvoederwaardering voor zowel praktisch als toekomstig gebruik.

List of presentations

Oral presentations

Gosselink, J.M.J., Poncet, C., Vérité, R., Dulphy, J.P., & Cone, J.W. (2000). Estimation of the duodenal flow of microbial nitrogen in ruminants based on the chemical composition of forages. Conseil scientifique de l'URH, 18 decembre 2000, INRA, Centre de Clermont-Ferrand-Theix, France.

Gosselink, J.M.J., Dulphy, J.P., Poncet, C. & Cone, J.W. (2002). Voorspelling van de *in vivo* schijnbaar verteerde organische stof in de pens van schapen met in-sacco-, enzymatische en in-vitro-methoden. 27e Studiedag Nederlandstalige Voedingsonderzoekers, 19 april 2002, Lelystad, the Netherlands, p 11 – 13.

Gosselink, J.M.J., Dulphy, J.P., Poncet, C., Tamminga, S. & Cone, J.W. (2003). *In vitro* and *in situ* prediction of rumen bypass protein in sheep. Proceedings IX World Conference on Animal Production, 26 – 31octobre 2003, Porto Alegre, Brasil, p 37.

Poster

Gosselink, J.M.J., Poncet, C. (2002). Effect of fresh and ensiled alfalfa and red clover on the microbial protein synthesis in the rumen of sheep. Abstracts 2002 Joint Meeting ADSA, ASAS, CSAS, Québec City, Canada, p 384

Curriculum Vitae

Jules Gosselink (baptismal names: Julius Maria Johannes) was born on the 9th of may 1965 in Lichtenvoorde. After he graduated from secondary grammar school in 1983, he started a study at the Agricultural College in Dronten. This study was finished in 1987, after which he worked in agriculture abroad and in the Netherlands. From 1988 till July 1995 he studied Veterinary Medicine at Utrecht University. For four years he was veterinary practitioner for dairy cattle in the north of the Netherlands. At the End of 1999 he learned about ruminant nutrition at ID-Lelystad as preparation for his PhD study. This study resulted in this thesis and took 4 years: 1.5 years in France at INRA and 2.5 years at Wageningen Institute of Animal Sciences (chair Animal Nutritrion), seconded at ID TNO Animal Nutrition (later: Animal Sciences Group of Wageningen UR, Nutrtion & Food).

Jules Gosselink (noms de baptême: Julius Maria Johannes) est né le 9 mai 1965 à Lichtenvoorde. Après avoir passé l'examen final de l' école secondaire en 1983, il commence ses études au Collège d'Agriculture de Dronten. Il termine en 1987 et travaille ensuite en relation avec la production agricole à l'étranger et aux Pays Bas. De 1988 à juillet 1995 il étudie la Médecine Vétérinaire à l'université d'Utrecht. Pendant quatre ans il est vétérinaire et travaille sur les vaches laitières dans le nord des Pays Bas. Durant l'automne 1999 il étudie la nutrition des ruminants à ID Lelystad afin de préparer une thèse. Ce document est le résultat de ce travail de thèse, qui a duré 4 ans : 1.5 an en France, à l'INRA de Clermont-Ferrand-Theix et 2.5 ans à Wageningen Institute of Animal Sciences (chaire de Nutrition Animale), attaché à ID TNO Animal Nutrition (appelé maintenant Animal Sciences Group of Wageningen UR, Nutrition & Food).

Jules Gosselink (doopnamen: Julius Maria Johannes) werd 9 mei 1965 geboren te Lichtenvoorde. Nadat hij het VWO diploma aan het Marianum te Groenlo in 1983 had behaald, heeft hij in Dronten een studie aan de Christelijke Agrarische Hogeschool gevolgd. Deze studie werd in 1987 afgerond en vervolgens heeft hij in binnen- en buitenland in de landbouw gewerkt. In 1988 begon hij de studie Diergeneeskunde aan de Rijksuniversiteit Utrecht en het dierenartsexamen werd in 1995 behaald. Hierna heeft hij 4 jaar als praktiserend dierenarts in de melkveehouderij van Noord-Nederland gewerkt. Eind 1999 heeft hij zich verdiept in de rundveevoeding bij ID Lelystad als voorbereiding op het promotieonderzoek. Dit onderzoek resulteerde in dit proefschrift en duurde vier jaar: 1.5 jaar in Frankrijk bij het INRA en 2.5 jaar bij Wageningen Institute of Animal Sciences (leerstoelgoep Diervoeding), gedetacheerd bij ID TNO Diervoeding (later: Animal Sciences Group van Wageningen UR, Voeding).

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