Evaluation of the Nutritive Value of Mediterranean Roughages

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Evaluation of the Nutritive Value of Mediterranean Roughages

Results and researches carried out within the
CAMAR EC/DG.VI Programme, contract 8001-CT90-0022
titled "Evaluation of Local Forage Resources
in the Mediterranean Region"

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Foreword

This issue of Options Méditerranéennes presents a research programme coordinated by the International Centre for Advanced Mediterranean Agronomic Studies through the Mediterranean Agronomic Institute of Zaragoza. This programme was carried out from 1991 to 1994 with the purpose of refining the biological and chemical methods used for making a reliable evaluation of the feeding value of Mediterranean-climate forages. This study was conducted within the framework of the CAMAR programme, financed by the European Union and successfully developed thanks to the efforts of seven research teams belonging to six countries of the European Union.

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The fodder resources available in the Mediterranean region have a chemical composition that depends on the climatic conditions of the countries concerned. This makes them different from the well-studied forages of the temperate humid areas. In particular, aridity, that is associated with high temperatures, increases the content of polyphenols, especially tannins that are likely to modify the transformation of nitrogen matter into nutrients.

Therefore it is appropriate to seek the best-adapted methods for predicting how animals can convert the forages in order to take maximum advantage of them and also to better use local resources and hence favour the farm.

Initially, a comparison was made of the different methods that would determine ingestibility, energy and nitrogen values of the forages with the purpose of showing which methods would be both reliable and easy to use in Mediterranean plants.

Special emphasis was placed on:

(i) Simplify the methods that enable us to determine the forage-takings of herbivores in polyphytic vegetation.

(ii) Identify the different categories of polyphenols according to their action on forage conversion.

(iii) Point out the particular characteristics involved in the use of nitrogen matter in Mediterranean forages.

Through this work it has been possible to:

(i) Show the interest of the "gas test" that is simple and repeatable for determining the feeding value of forages and by-products of the Mediterranean region.

(ii) Determine the composition and feeding value of almost 100 forages of which little was previously known.

The methods chosen were later tested to determine the protocol by the four chains of analysis which, besides the teams in the programme, involved four laboratories situated in North Africa: Institut National d'El Harrach, Algeria; Institut Agronomique et Vétérinaire Hassan II, Rabat, Morocco; Institut National Agronomique de Tunis and Ecole Supérieure d'Agriculture de Mateur, Tunisia, thanks to the support of the International Centre for Advanced Mediterranean Agronomic Studies.

As a conclusion of all the trials carried out, a compilation was made that included the recommended methods under Mediterranean conditions, the content of phenolic substances, intake, digestibility and use of nitrogen matter in Mediterranean forages. For each technique the conditions and limitations of its use are described.

We trust we have contributed, through this project, towards a better estimation of the local forage possibilities to enhance animal production in Mediterranean countries.

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Avant-propos

Ce numéro d'options méditerranéennes est consacré à la présentation d'un programme de recherches coordonnées par le Centre International de Hautes Etudes Agronomiques Méditerranéennes à travers l'Institut Agronomique Méditerranéen de Saragosse. Conduite de 1991 à 1994, dans le but d'affiner les méthodes biologiques et chimiques permettant d'apprécier de façon plus fiable, la valeur alimentaire des fourrages, produits en climat méditerranéen, cette étude réalisée dans le cadre du programme CAMAR, financé par l'Union Européenne, a pu être menée à bien grâce aux efforts de sept équipes de recherches appartenant à six pays de l'Union Européenne.

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Les ressources fourragères disponibles dans la région méditerranéenne ont une composition chimique qui dépend des conditions climatiques des pays concernés. Cela les rend différentes de ce qui est bien connu dans la zone tempérée humide. En particulier l'aridité, associée à des températures élevées, augmente les teneurs en polyphénols et en particulier en tanins qui sont susceptibles de modifier la transformation des matières azotées en nutriments.

C'est pourquoi il convient de rechercher les méthodes les plus adaptées permettant de prévoir l'utilisation alimentaire que les animaux peuvent faire de ces fourrages afin d'optimiser leur valorisation et aussi de mieux utiliser les ressources locales au profit de l'élevage.

Dans un premier temps la comparaison entre les différentes méthodes permettant de déterminer l'ingestibilité, les valeurs énergétiques et azotées des fourrages a été réalisée afin de mettre en évidence celles qui allient fiabilité et facilité d'emploi dans le cas des plantes méditerranéennes.

Une considération spéciale a été attachée à :

(i) Simplifier les méthodes permettant de connaître le prélèvement des herbivores dans la végétation polyphyte.

(ii) Identifier les différentes catégories de polyphénols en fonction de leur action sur l'utilisation des fourrages.

(iii) Préciser les particularités de l'utilisation des matières azotées des fourrages méditerranéens.

Ce travail a permis de :

(i) Mettre en évidence l'intérêt du "gaz test" qui s'avère une méthode simple, répétable, permettant de préciser la valeur alimentaire des fourrages et des sous-produits dans la zone méditerranéenne.

(ii) Connaître la composition et la valeur alimentaire de près d'une centaine de fourrages mal connus jusqu'à ce jour.

Les méthodes choisies ont été testées par la suite pour préciser leur protocole lors de quatre chaînes d'analyses impliquant en plus des équipes du programme, quatre laboratoires situés en Afrique du Nord : Institut National d'El Harrach, Algérie ; Institut Agronomique et Vétérinaire Hassan II, Rabat, Maroc ; Institut National Agronomique de Tunis et Ecole Supérieure d'Agriculture de Mateur, Tunisie, grâce à l'appui du Centre International de Hautes Etudes Agronomiques Méditerranéennes.

En conclusion des travaux réalisés un recueil a été préparé qui comprend les méthodes recommandées pour déterminer dans des conditions réalisables en zone méditerranéenne, la teneur en substances phénoliques, l'ingestion, la digestibilité et l'utilisation des matières azotées des fourrages méditerranéens. Pour chaque technique sont précisées les conditions et les limites d'emploi.

Aussi nous espérons avoir contribué à travers ce projet à une meilleure estimation des possibilités fourragères locales pour conforter les productions animales dans les pays méditerranéens.

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Summary

This research, funded by a European Union contract, was carried out by seven laboratories from six European countries, whose objective was to improve knowledge of the nutritive value of forages and by-products of the Mediterranean region. In order to achieve this goal, researchers had to be provided with adapted, reliable and inexpensive assessment methods. In fact, it was soon realized that the techniques commonly used for forages grown in wet temperate climates were not always applicable to obtain reliable results for forages produced in the Mediterranean region, which is characterized by a hot and arid climate. In particular, the cell wall structure is different, and the presence of polyphenolics such as tannins alters intake and digestion of the main constituents of Mediterranean forages and by-products.

The work placed special emphasis on the comparison between the main biological and chemical methods known, to determine which of them were best adapted to the forages, studied. In a second phase, work was aimed at improving these methods to make them more efficient and, at the same time, to maximize their reproducibility and to minimize their cost.

This research work has focused on the following:

(i) Feeding behaviour of animals, to gain knowledge on bite numbers on rangelands.

(ii) Chemical and biological methods.

(iii) The specific problem of polyphenolics.

The possibilities and conditions for using the main methods available were evaluated. Of these methods, the gas test method seems to be the most promising, being simple to use, reproducible and allowing the prediction of level of intake and apparent digestibility of forages, as well as assessing the impact of phenolic compounds.

The level of intake and digestibility measured *in vivo* on small ruminants (sheep and goats) were taken as a reference to test the reliability of *in vitro* and *in situ* methods used for assessment under inexpensive conditions.

The implementation of chains of analyses involving the seven teams in the project and four laboratories from Algeria, Morocco and Tunisia allows the feasibility and reproducibility of the selected methods to be determined. These are described in the second part of the document.

Résumé

Cette recherche financée par un contrat de l'Union Européenne a été conduite par sept laboratoires de six pays européens et a eu pour objectif d'améliorer la connaissance de la valeur nutritive des fourrages et sous-produits de la région méditerranéenne. Pour atteindre ce but il fallait mettre à la disposition des chercheurs des méthodes d'évaluation adaptées, fiables et peu coûteuses. En effet il est vite apparu que les techniques couramment utilisées pour les fourrages cultivés en climat tempéré humide n'étaient pas toujours applicables pour avoir des résultats fiables sur les fourrages produits dans la zone méditerranéenne qui se caractérisait par un climat chaud et aride. En particulier la structure des parois végétales est différente et la présence de polyphénols comme les tannins modifie l'ingestion et la digestion des principaux constituants des fourrages et sous-produits méditerranéens.

Les travaux ont porté en particulier sur la comparaison des principales méthodes chimiques et biologiques connues pour déterminer celles qui étaient les mieux adaptées pour les fourrages concernés. Dans un deuxième temps les actions ont été dirigées à améliorer les méthodes pour les rendre plus efficaces tout en maximisant leur reproductibilité et en minimisant leur coût.

Les travaux ont concerné les points suivants :

(i) Le comportement alimentaire des animaux afin de mieux connaître les prélèvements effectués sur le parcours.

(ii) Les méthodes chimiques et biologiques.

(iii) Le problème spécifique des polyphénols.

Les possibilités et les conditions d'utilisation des principales méthodes disponibles ont été évaluées. Parmi celles-ci le "gaz test" apparaît le plus prometteur, il est simple d'emploi, reproductible et permet de prévoir l'ingestibilité et la digestibilité apparente des fourrages ainsi que d'évaluer l'impact des composés phénoliques.

L'ingestibilité et la digestibilité mesurée *in vivo* sur petits ruminants (ovins - caprins) ont été considérées la référence pour tester la fiabilité des méthodes *in vitro* et *in situ* permettant d'obtenir des évaluations dans des conditions économiques.

La réalisation de chaînes d'analyses concernant les sept équipes du projet et quatre laboratoires d'Algérie, Maroc et Tunisie, permet de juger de la faisabilité et de la reproductibilité des méthodes choisies. Ces dernières sont proposées dans la deuxième partie de ce document.

Introduction

A project funded by the European Union has made it possible for seven teams to work together from September 1991 to September 1994 in order to improve our knowledge on the nutritive value of Mediterranean forages.

1. Teams

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2. Objective

The Mediterranean region is characterized by a semi-arid climate due to the fact that in some years summer drought expands over spring and autumn, in addition to high temperatures. As a consequence the vegetation is steppe-like. Nevertheless ruminant animal productions are traditionally based on grazing complemented by browse and straw left on the fields.

Small ruminants have proved to perform better than cattle, especially if the latter are derived from breeding.

This situation exists at least partially in five European Union countries (France, Greece, Italy, Portugal and Spain).

In order to justify the maintenance of an extensive livestock system in the areas involved, with no competition against the animal production systems of the regions North of the European Union, it is convenient to gain a better knowledge of the nutritive value of Mediterranean forages.

It is for this purpose that seven teams belonging to six European countries have joined efforts to gain better knowledge of the nutritive value of Mediterranean forages. The aim is to know more accurately the nutritive value of plant species specific of the Mediterranean area but also to evaluate the effects of climate on the composition of species known in the rainy temperate zone and their consequences on animal utilization.

Using first of all the methods commonly employed to study the forages produced in the rainy temperate zone very soon proved inadequate. Indeed the plants developing in hot arid areas have a

cell wall composition different from temperate forages. An example of this is that dosage of crude cellulose by the Weende method does not provide an evaluation of digestibility for these forages. Furthermore, the polyphenolics as well as tannin content depress microbial activity in the digestive tract as this is limiting for energy utilization of forages, and this is also true for nitrogen matter provided.

This is why research was carried out taking as a priority the improvement of methods for assessing Mediterranean forage and by-product nutritive value.

Four factors of forage nutritive value have been especially studied:

(i) Feeding behaviour and forage takings by animals on rangelands.

(ii) Selecting the chemical and biological methods most adequate to predict forage value.

(iii) Polyphenolics problem, especially important for shrubs.

(iv) Assessment of nitrogen value of forages.

3. Main results

3.1. Feeding behaviour

It is quite important to know accurately what is taken by the animal on multi-plant rangelands, in quantity and quality. The diverse methods available have disadvantages so that observations are not always reliable due to their more or less artificial aspect. The work carried out by R. Cordesse shows that samples of rumen content, if some corrections are introduced, prove to be the most reliable. They suppress human presence on rangelands, which could alter animal behaviour.

On the other hand the measurement of level of intake on forages cut and given to the animal, does not respond to reality.

3.2. Selection of chemical and biochemical methods

The work carried out in Udine (P. Susmel), Salonica (A. Nastis), and Santarem (J.M.C. Ramalho-Ribeiro) laboratories from reference measurements made *in vivo* show that it is possible to assess intake and digestibility of conventional forages made up of grasses and pulses. However it is necessary to estimate cell wall composition by van Soest fractioning method. Nevertheless, the *in situ* degradation techniques with nylon bags have proved to be more accurate than *in vitro* techniques (Tilley and Terry, 1963).

It is not possible to reliably estimate the feeding value of browse with these techniques.

It appears that the gas test method proposed by Ørskov and Khazaal (Aberdeen, Scotland) is the most adequate for the evaluation of intake and level of intake for all Mediterranean forages and by-products.

3.3. Polyphenolics study

The feeding value of browse is not only limited by lignin, but also by tannin-like polyphenolics that can even be toxic in some cases. This phenomenon applies to many forage resources in Mediterranean rangelands.

Various fractions have been dosed: total polyphenolics, soluble tannins and condensed tannins, their effects on cell wall digestibility and nitrogen utilization have been measured on some fifty samples from the main shrubs browsed by animals in the Mediterranean region. It appears that

condensed tannins are the major antinutritional factors. For their determination, the gas test is also more reliable than the *in situ* technique with nylon bags, probably due to the mitigation of antinutritional effects of condensed tannins introduced in small quantities in the nylon bag.

The treatment applied to these forages with tannin-blocking substances has been successful and in particular it does not disturb fermentations in the digestive tract.

3.4. Assessment of nitrogen value of forages

In ruminants, the utilization of feed nitrogen matters depends on their degradability in the rumen, so that the assessment can be made of the proportion likely to be broken down into amino acids in the small intestine and the proportion available for microbes to synthesize microbial proteins well-balanced in essential amino acids.

In most cases and notably to determine PDI value of forages in France, feed nitrogen matter degradability is assessed by means of the *in situ* technique with nylon bags introduced in the rumen of cows fitted with a permanent rumen cannula.

The assays show it is possible to reduce the cost of this technique by using fistulated sheep which are more versatile. Indeed it has been proved that, on six Mediterranean forages and by-products, the degradation curves between 2 h and 48 h are comparable and provide reliable data on the nitrogen value of samples studied.

3.5. Proposal of methods adapted to the assessment of nutritive value

This research has been complemented by a series of assays aiming at harmonizing the methods used in the different laboratories, carried out by four chains of analyses for chemical determination of forages and especially cell wall fractioning by van Soest method, the *in situ* methods with nylon bags and the gas test.

This enables us to propose a collection of recommended methods that should provide reliable results which may be compared between laboratories and to carry out syntheses on many values.

References

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First Part

Criteria for Selecting Assessment Methods

Criteria for selecting assessment methods

1. Introduction

The countries surrounding the Mediterranean basin have extensive forage resources, including natural and semi-natural vegetation, together with the more usual agricultural crops, such as lucerne, clover and grass. These resources may be utilized in various ways, such as extensive or intensive grazing with animals (generally sheep and goats) or by cutting and carting to housed or corralled animals. Given the size of the area, it is not surprising that a vast number of plant species are involved, each with its own range of stages of growth, nutritive value, yield and possible presence of antinutritive substances. Many countries also practise extensive grazing under trees (both spontaneous and planted) and in many places this contributes not only a useful forage resource but also helps to reduce the risk of fire in the hot summer months.

In order to be able to improve and develop the agriculture in these regions, it is important to know or be able to predict the nutritive value of the feeds consumed and have an idea of the quantity consumed. This latter aspect can be particularly difficult to assess as many of the plants involved are not simply grazed but nibbled or browsed by animals such as goats, so then the problem lies in determining what is the digestibility of the fraction of the plant consumed.

2. Feeding behaviour

The ability of animals to choose plants amongst those present on their territory depends upon a number of factors. The first parameter for choice refers to the animals themselves. They are often classified into two categories, grazers or browsers, according to their consuming preferably forages belonging to the herbaceous or lignified strata. The second parameter for variability of feed choice, are plant heterogeneity and variations of chemical composition between the different phenologic stages, as well as climatic characteristics (Holechek *et al.*, 1981; Senft *et al.*, 1987).

The techniques for the assessment of feed choice are diverse and more or less efficient.

Visual observations based on determining the time spent for consuming a certain plant type are the easiest to conduct. Therefore the species and plant fractions apparently consumed can be listed. But they remain poor for providing sufficient data on the relative proportion of each plant type in the diet (Guérin, 1987).

For animal species with a tendency to gather their feeds and to be selective as regards their feed take (camels, goats and sheep), the bite method has been used (Bourbouze, 1980). The weight of feeds consumed with each bite is assessed either by hand picking as a simulation of biting (Raymond, 1954; Meuret *et al.*, 1985), or by measuring a sample provided directly (Dumont, 1991). This method is very time-consuming and requires great concentration. Besides, the observer could alter animal behaviour if staying too closely. In the case of Mediterranean pastures, the plant heterogeneity and height of the lignified stratum is too great and therefore this assessment of the vegetation consumed does not fully provide accurate results (Guérin, 1987; Penning and Treacher, 1989).

Other methods use analysis of oesophagus bolus samples, or rumen or faeces contents. In all cases, these samples must be carefully prepared. Oesophagus bolus and rumen contents collected after the main meal and before rumination, have the advantage of having undergone little mastication. The plant fragments observed are easy to identify. On the contrary, in faeces samples, these fragments are very altered due to digestion and sometimes are difficult to identify.

On bushy rangelands, the methods using devices as oesophagus fistula give random results and are dangerous for the animal who bears it.

There are diverse methods for counting total particles taken at rumen or oesophagus bolus level (Galt *et al.*, 1969; Smith and Shandruk, 1979). Guided by the method described by Galt *et al.* (1969),

we have defined an identification technique for feed choice to be used in a bushy heterogeneous environment. Unlike direct observation methods, the presence of the operator in the field is restricted to taking the rumen sample. This makes this task easier and reduces disturbances to the animals.

This method requires that animals should be fitted with a rumen cannula. The extractions of rumen content are carried out at the end of the main meal and before the beginning of rumination. So that the feeds collected from the last meal have not yet undergone an intense mastication but are simply influenced by their presence in the rumen.

After homogenization of rumen content, a sample of about 1 kg is taken, homogenized and 150 to 200 g is kept. The rest is given back to the animal.

The samples are washed several times under running water through 3 sieves with decreasing hole sizes from 10 mm to 2.5 mm. A security sieve with 1.25 mm hole size is used to collect elongated particles. Some still exhibit the green colour of plants just swallowed. The content of the sieve is sorted several times on the 2.5 mm sieve to retain these elongated particles still green from the last meal.

All the plant particles isolated during the sorting are identifiable by means of a previously established herbarium.

The sorting itself consists in grouping together the particles according to plant species and at the same time separating leaves, woody parts, leaf blades. They are then dried at 60°C during 3 days, in a ventilated oven, and weighed afterwards.

The woody parts with no bark are not kept for this sorting operation as they have undergone rumination.

Corrective measures are introduced in these samples to take into account the suppression of soluble matters when in contact with saliva, rumen liquor and washing waters. The rapidly soluble fraction of plants has been measured using the *in sacco* method on fresh material coarsely chopped, maintained *in situ* for 4 and 8 hours. For woody samples Tilley and Terry (1963), method was used limited to a 24 hours fermentation time. The *in sacco* method could not be used as some woody parts perforate the bag fabric used with this technique, distorting measurements.

The corrective factors used, in order to account for the matters solubilized in the rumen and during washings, are 20% for grasses, 18% for kermes oak leaves, 10% for juniper (*Juniperus oxycedrus*), 2% for woody parts. For plants randomly consumed by animals and that make up only a small proportion of the total, we are using a global value of 10%.

3. Nutritive value

The most accurate determination of the nutritive value (as reflected by digestibility, levels of intake and production) of animal feeds can be achieved by feeding trials (*in vivo)*. However, the *in vivo* approach is considered to be expensive (cost of labour, feeds, animals) and time consuming. Therefore, prediction of both daily intake and digestibility of roughages by ruminants using simple, reliable and cheap techniques is very important in animal nutrition. As an alternative to *in vivo* studies, several laboratory techniques have been developed for the purpose of predicting animal performance (e.g., daily intake, digestibility) and assessing the nutritive value of feedstuffs. The application of these techniques in animal nutrition studies depends to a large extent on their reliability, simplicity and the cost involved in using these techniques.

Amongst the laboratory techniques that are widely used in feed evaluation at present are the fibre analysis (van Soest and Wine, 1967), the two-stage *in vitro* method (Tilley and Terry, 1963) and the rumen incubation *in situ* method (Ørskov, 1989; Ørskov and Ryle, 1990). The gas production technique *in vitro,* as described by Menke and Steingass (1988) to predict metabolizable energy (ME) content has still not been widely used. This may be because it requires in addition to the determination of gas production after 24 h incubation, measurement of the concentration of crude protein (CP), crude fat, crude fibre and ash in the feed. However, as the number of species and type

of feeds investigated was quite variable and large, it would therefore be better to divide all the investigated feeds into two main groups:

(i) Conventional forages, consisting of species of *leguminous* or *graminaceous* hays harvested at different stages of growth. This type of feeds are commonly grown and used in animal production systems that are considered to be intensive.

(ii) Unconventional forages, consisting of fodder of multipurpose trees and shrubs of browse at different stages of growth. This type of feed is commonly used under extensive animal production systems where the availability and the quality of feeds change during the year. Studying this type of forages it became evident that, due to the presence of phenolics-related antinutritive compounds such as tannins, the nutritive value of the unconventional forages could not be accurately determined using conventional chemical or biological assays. Therefore a great deal of effort was put into developing new techniques in order to assess the nutritive value of these unconventional forages more accurately.

3.1. Conventional forages

3.1.1. Material and methods

Three harvesting stages (early bloom EB, mid bloom MB or in seed IS) made from lucerne (*Medicago sativa*), sweet clover (*Melilotus segetalis*), Persian clover (*Trifolium resupinatum*), Rye (*Secale cereale*), Triticale (*Triticale hexaploid*), oat (*Avena sativa*) and a pre-bloom (PB) Italian ryegrass (*Lolium multiflorum*). The hays were grown without irrigation at Santarem, Portugal. After harvest, the hay was left for one week in the field to dry.

Thus, data of the 10 graminaceous hays and the 9 leguminous hays were combined and the ability of the fibre analysis, the 2 stages *in vitro* digestibility (Tilley and Terry, 1963), the *in situ* DM degradation or the gas production techniques to predict daily intake (g DM/kgW$^{0.75}$) and *in vivo* DM digestibility (DMD) of 19 leguminous and graminaceous hays fed to sheep were tested (Dentinho *et al.*, 1994; Khazaal *et al.*, 1995).

3.1.2. Results

The concentrations (g/kg DM) of NDF, ADF and ADL of the hays were generally increased with maturity whereas that of CP decreased. The stage of maturity of the hays was well reflected in their levels of intake (g DM/kgW$^{0.75}$) but not in their apparent *in vivo* DMD (g/kg DM). Apparent *in vivo* digestibility was negatively and significantly ($P<0.05$) related to ADF ($r=-0.57$) and ADL ($r=-0.65$) concentrations only. On the other hand, intake was significantly ($P<0.01$) related with NDF ($r=0.61$) and CP ($r=0.73$) only. However, the *in vitro* DMD was not related ($P>0.05$) to intake ($r=0.45$) or to *in vivo* DMD ($r=0.44$).

The *in vivo* DMD was significantly ($P<0.05$) related with gas production at 24-96 h ($r=0.68$-0.76) incubation. On the other hand intake was significantly ($P<0.05$) related to volume of gas at 12 h incubation ($r=0.54$) and to the rate constant (c) of gas production ($r=0.52$). As the hays were incubated for a longer period (i.e., 48-96 h), the difference in gas production between the leguminous and the graminaceous hays and also the difference between the three harvesting stages of each hay became smaller.

The results of simple or multiple regression between intake or apparent DMD *in vivo* of the hays and either fibre components, *in vitro* DMD, or characteristics of gas production are shown in Table 1. Using NDF, ADF and ADL in a multiple regression resulted in good accuracy in predicting both intake and *in vivo* DMD. The prediction was further improved when CP was included as an additional factor. However, using the (a+b) and (c) of gas production, prediction of *in vivo* DMD ($r=0.73$; $P<0.01$) was more accurate than that of intake ($r=0.56$; $P<0.05$). The inclusion of CP as an additional factor with the (a+b) and (c), resulted in a larger improvement in predicting intake and apparent *in vivo* DMD from gas production and apparent *in vivo* DMD from gas production in comparison with nylon bag degradation.

Table 1. Prediction of voluntary daily intake (g DM/kgW$^{0.75}$) or apparent *in vivo* dry matter digestibility (DMD) (g/kg DM) of 19 hays [3 harvesting stages of legumes (Lucerne, sweet clover and Persian clover) (Khazaal *et al.*, 1995) or graminae (triticale, rye and oat) and a ryegrass] by sheep from fibre components, *in vitro* DMD (Tilley and Terry, 1963), or from characteristics of cumulative *in vitro* gas production or progressive *in situ* DM degradation over 96 h period generated from the equation p = a+b (1-e^{-ct}). From Khazaal *et al.* (1995)

Y	Technique	Equation and factors used	r	Sig.	RSD
Intake	Fibre components	112 – 0.084(NDF)	0.615	0.005	9.2
		83 – 0.051(ADF)	0.319	0.183	11.0
		62 – 0.022(ADL)	0.054	0.830	11.6
		116 – 0.075(NDF) -0.041(ADF) + 0.134(ADL)	0.634	0.047	9.6
		32 – 0.063(NDF) + 0.13(ADF) – 0.22(ADL) + 0.22(CP)*	0.793	0.005	7.8
	Tilley and Terry	21 + 0.070(*in vitro* DMD)*	0.444	0.056	10.4
	Gas production *in vitro*	73.7 – 0.29(a+b)	0.161	0.510	11.5
		2.4 + 0.52(a+b) + 533(c)*	0.564	0.046	9.9
		-2.6 + 0.49(a+b) + 339(c)* + 0.17(CP)*	0.798	0.001	7.4
		3.5 + 0.63(a) + 0.50(b) + 532(c)*	0.558	0.123	10.3
	DM degradation *in situ*	32.6* 0.40(a+b)	0.356	0.134	10.9
		36.5 + 0.08(a+b) + 0.62(A)	0.452	0.160	10.7
		9.9 + 0.40(a+b)* + 408(c)***	0.847	0.000	6.3
		13.5 – 0.31(a+b) + 328(c)* + 0.066(CP)	0.862	0.000	6.3
		9.5 + 0.39(A) + 0.43(B) + 411(c)***	0.847	0.000	6.5
DMD	Fibre components	671 – 0.188(NDF)	0.214	0.378	72.7
		815 – 0.584(ADF)	0.568	0.011	61.2
		676 – 1.76(ADL)	0.649	0.003	56.6
		695 – 0.026(NDF) – 0.017(ADF) + 1.7(ADL)	0.651	0.036	60.1
		618 – 0.015(NDF) + 0.14(ADF) – 2.03(ADL) + 0.20(CP)	0.654	0.080	62.0
	Tilley and Terry	306 + 0.45(*in vitro* DMD)	0.446	0.055	66.6
	Gas production *in vitro*	190 + 8.3(a+b)***	0.708	0.001	52.5
		29 + 10.2(a+b)*** - 1199(c)	0.733	0.002	52.1
		15 + 10.1(a+b)*** + 623(c) + 0.51(CP)	0.778	0.002	49.7
		-17 + 14.9(a) + 10.9(b)** + 1559(c)	0.738	0.007	53.4
	DM degradation *in situ*	145 + 5.9(a+b)***	0.824	0.000	42.1
		136 + 6.6(a+b)*** - 1.3(A)	0.830	0.000	42.8
		161 + 5.9(a+b)*** - 284(c)	0.828	0.000	42.9
		140 + 6.43(a+b)*** + 175(c) – 0.37(CP)	0.841	0.000	42.8
		143 + 5.40(A)** + 6.6(B)*** - 190(c)	0.839	0.000	43.1

A: Washing loss (water soluble fraction); B: insoluble but fermentable matter [B = (a+b) - A]; a, b and c: constants in the exponential equation p = a+b (1-e^{-ct}), a+b = potential DM degradation, c = rate of DM degradation

r: Correlation coefficient; Sig.: Significance of the portion of variation in the data explained by the model; RSD: Residual Standard Deviation

Significance of contribution of additional terms to the model: *P<0.05, **P<0.01 and ***P<0.001

3.1.3. Discussion

The best predictions of both daily intake and apparent digestibility of a combination of different plant types were obtained using characteristics of *in situ* DM degradation. Due to the problem of protein fermentation in the gas test, prediction of intake and apparent digestibility from characteristics of gas production were less accurate than from DM degradation. However, and despite that, the characteristics of gas production were better predictors of intake and apparent DMD than any of the chemical components or the *in vitro* DMD (Tilley and Terry, 1963). The results clearly show that accurate prediction of both intake and digestibility can be achieved simply from the characteristics of degradation of feeds.

3.2. Unconventional forages

In many parts of the world, browse (shrubs and tree leaves) is widely available and traditionally used by farmers. For instance, in Northern Africa, browses constitute 60-70% of rangeland production and 40% of the total availability of animal feeds (Devendra, 1989). Animal production from the extensive systems of Mediterranean Europe has been contributing and will continue to contribute to the overall animal production in Europe. Improving the efficiency of the system will help it to be more competitive and at the same time improve the sources of income for farmers in rural communities.

Variations in the nutritive value of browsing species have been observed (Wilson, 1977; Nastis, 1985) and have also been found to be affected by maturity (Papachristou and Nastis, 1990). In forages, the decline in digestibility with maturity has been attributed to increased lignification (van Soest, 1987) or to changes in chemical composition, mostly of the stem (Nordkvist and Aman, 1986). Changes in fibre components also affect the nutritive value of browse, however polyphenolic compounds such as tannins have been considered to have a more detrimental effect (Makkar *et al.*, 1988, 1991). Ruminants fed with diets rich in tannins showed reduced feed intake, depressed protein and fibre digestibility, increased nitrogen excretion in faeces (Nastis and Malechek, 1981; Kumar and Vaithiyanathan, 1990) and sometimes toxicity symptoms (Lohan *et al.*, 1983; Barry and Duncan, 1984).

As a result of the increasing recognition of the role that browse can play in ruminant nutrition, changes in chemical composition or nutritive value of browse has been extensively studied in Tropical Africa and South East Asia. However, information on Mediterranean browse is very limited. In the Mediterranean area, Kermes oak (*Q. coccifera*) occupies large areas of the rangelands but the most important deciduous browsing species in Greece are *C. Duinensis* and *F. ornus* which are grazed mainly from spring until autumn (Papachristou and Nastis, 1990). Under this project the first objective was to determine the most efficient methods to assess nutritive value of local forages in the Mediterranean area and to investigate changes in nutritive value with emphasis on their polyphenolic compounds. Based on such information, it may be possible to improve the efficiency and the productivity of such ruminant production systems by making them more based on available feeds other than cereals or concentrates which could be used for human nutrition.

3.2.1. Material and methods

The samples were mainly of three sources: Greece, Spain and Tunisia (Table 2). Most of the browse consisted of leaves of indigenous Greek browse that were grown in a subhumid Mediterranean zone (*Quercefalia pubescentis*) in Macedonia (650 m elevation; minimum annual temperature 13°C; rainfall 630 mm). The Spanish samples were collected from different Mediterranean zones. Thus *A. campestris* and *H. stoechas* were collected from a subhumid zone (*Genisto - baeticae - Juniperetum - nanae;* average temperature 8.4°C; rainfall 708 mm; elevation 1868 m). *S. chamaecyparissus, T. polium, Q. rotundifolia, R. officinalis* and *A. decorticans* were collected from a dry or subhumid zone (*Paeonio - Quercetum - rotundifoliae*; average temperature 11-12°C; rainfall 582-605 mm; elevation 1050-1510 m). The samples were harvested by hand and dried in an oven at 50-60°C for 48 h followed by storing in the dark at room temperature until they were used.

Within the framework of this project, feed samples which had been tested for *in vivo* dry matter digestibility and intake were subjected to chemical analyses, *in vitro* and *in situ* assays in order to study the possibility of their prediction.

Digestibility and intake trials were performed at the laboratories of origin, using, where possible, standard protocols; goats and sheep were used to test the shrubby species. a) *Fraxinus ornus, Carpinus duinensis* and *Quercus pubescens* were used for *in vivo* trials in Greece. They were grown in a subhumid Mediterranean zone (*Quercetalia pubescentis*) in Macedonia (650 m elevation; minimum annual temperature 13°C; rainfall 630 mm). b) *Acacia salicina, Robinia pseudoacacia, Atriplex nummularia* and *Anthyllis cytisoides* were tested in Spain. c) Digestibility and intake of mature (November) *Quercus coccifera* was determined in France.

Table 2. Description and characteristics of the browse studied

Scientific name	Country of origin	English/ native name	Harvesting period	General information
Philleria media	Greece	-	Spring 91	Leaves. Sclerophyllous evergreen shrub
Cistus incanus	Greece	Rock rose	June 91	Leaves. Woody shrub
Cistus incanus	Greece	Rock rose	June 93	
Cistus incanus	Greece	Rock rose	Sept. 93	
Arbutus andrachnoides	Greece	-	February 92	Leaves. Hybrid of Arbutus spp.
Arbutus andrachnae	Greece	-	Spring 91	Leaves. Evergreen shrubby species
Arbutus andrachnae	Greece	-	October 91	
Arbutus andrachnae	Greece	-	June 93	
Arbutus unedo	Greece	Strawberry tree	Summer 91	-
Arbutus unedo	Greece	Strawberry tree	November 91	
Arbutus unedo	Greece	Strawberry tree	September 93	
Carpinus duinensis	Greece	-	June 91	Leaves. Deciduous shrub
Carpinus duinensis	Greece	-	October 91	
Carpinus duinensis	Greece	-	September 92	
Quercus coccifera	Greece	Kermes oak	June 91	Leaves. Sclerophyllous evergreen shrub
Quercus coccifera	Greece	Kermes oak	October 91	
Fraxinus ornus	Greece	Manna ash	June 91	Leaves. Deciduous shrub
Fraxinus ornus	Greece	Manna ash	October 91	
Fraxinus ornus	Greece	Manna ash	Sept. 92	
Robinia pseudoacacia	Greece	Black locust	Sept. 92	Grazed leaves (GL)
Robinia pseudoacacia	Greece	Black locust	Sept. 92	Ungrazed leaves (UL)
Robinia pseudoacacia	Greece	Black locust	Sept. 92	Ungrazed branches (UB)
Quercus pubescens	Greece		June 1993	
Quercus pubescens	Greece		July 1993	
Quercus pubescens	Greece		September 1993	
Juniperus oxycedrus	Greece		June 1993	
Juniperus oxycedrus	Greece		September 1993	
Artemisia campestris	Spain	Escobilla parda	July 91	Fine stems + leaves + flowers
Helichrysum stoechas	Spain	Boja blanca	July 91	Fine stems + leaves + flowers
Santolina chamecyparissus	Spain	Abrota hembra	July 91	Fine stems + leaves + flowers
Teucrium polium	Spain	Moradu	July 91	Fine stems + leaves + flowers
Adenocarpus decorticans	Spain	Rascaviejas	November 91	Fine stems + leaves
Quercus rotundifolia	Spain	Encina	November 91	Fine stems + leaves
Rosmarinus officinalis	Spain	Rosemary/Romero	November 91	Fine stems + leaves
Quercus coccifera	France	Kermes oak		
Acacia cyanophylla	Tunisia	A. Orangewattle		
Acacia cyanophylla	Tunisia	A. Orangewattle		
Artemisia campestris	Tunisia	Sagewort wormwood		
Artemisia herba alba	Tunisia			
Atriplex halimus	Tunisia	Salt bush		
Ceratonia siliqua	Tunisia	Carob		
Cistus libanotus	Tunisia			
Cistus salvigolus	Tunisia			
Globula alypum	Tunisia			
Juniperus phoenicea	Tunisia			
Opuntia ficus indica	Tunisia	Barbary fig		
Phyllirea angustifolia	Tunisia			
Pistacia lentiscus	Tunisia			
Rosmarinus officinalis	Tunisia	Rosemary		

The samples (Table 2) were milled through a 1.0 mm screen and were analysed for DM, ash, fibre composition according to van Soest and Wine (1967) and for crude protein (CP) as nitrogen (AOAC, 1990) multiplied by 6.25. Total extractable phenolics (TEPH) (Julkunen-Tiitto, 1985), tannins (TETa) (Makkar *et al.*, 1993), and condensed tannins as Vanillin-HCl (TECTa) (Broadhurst and Jones, 1978) or as extractable and total proanthocyanidins (TEPAs and TOPAs) (Porter *et al.*, 1986) were determined. Gas production from the samples was measured. For detailed description of the analyses, please refer to Khazaal *et al.* (1994, 1996) or to the reference book.

The presence of tannins that could form complexes with proteins and make them indigestible, poses the problem of nitrogen value of these forages. In order to propose a technique adapted to the Mediterranean region, we compared the degradability of nitrogen matters *in situ* by the nylon bag technique in cows and sheep, the latter being more easily used and cheaper in Mediterranean countries.

The total quantity of feeds distributed daily was 7 kg DM per cow and 1.45 kg DM per sheep distributed in 2 equal meals, at 8 and 17 h.

Six Mediterranean forages are being experimented on three cows and six sheep fitted with a rumen cannula. They are chopped in a hammer-chopper with a 1 mm or 5 mm grid. These feeds are durum wheat straw, strawberry tree, American broad bean pods (variety Mil), oak leaves (*Quercus coccifera* and *Q. pubescens*) and ash leaves (*Fraxinus ornus*). Durum wheat straw and strawberry tree are chopped with a 5 mm grid. Dehydrated lucerne and soyabean cake serve as control feeds.

The fabric used is Blutex T50 with 46 µm perforation size. A 15 cm side square is folded and welded on 3 faces. A double welding on the fourth face supports an eyelet on the part that does not contain the feed to be studied. The inner size is then 11 cm x 6 cm resulting in a relationship of feed quantity to exchange surface area of about 20 mg/cm^2. Weighings at each stage determine the feed DM quantity in each bag as well as the dry weight of finished bags. The DM in feeds and bags are determined by weighing samples placed in an oven for 48 h at 80°C. The finished bags are tied to a ballasted thread.

During every measurement, 6 kinetics points are observed, at 2, 4, 8, 16, 24 and 48 h. In each case, 6 replications were made. As regards cows, each replication was made with two bags. One bag only is enough for sheep.

The bags are kept in a dry place, in the dark and with ambient temperature. They are implanted 8 h. before the meal. Once taken out, the bags are immediately rapidly washed under tap water. Then they are frozen at -15°C (to inhibit micro-organisms and their enzyme activity). The bags are washed 3 to 5 times for 5 mn in a washing machine until rinsing water is clear, which is called conventional washing. During this experiment, a new washing system has also been tested: the bags are put into the stomacher after a conventional washing. The stomacher is used for a better separation of microbial particles of plant fragments, as they can cause an error of overestimation during the dosification of residual nitrogen matters in the case of initially low-nitrogen forages.

The stomacher protocol is as follows: after conventional washing, the bags are deprived of their eyelets and placed 3 at a time into a larger bag with little water. They are then subjected for 7 mn to the action of this machine under slow mode (a stronger treatment would deteriorate several bags). The eyelets must be conserved and kept with the corresponding bags. The stomacher bags must also be regularly replaced to avoid their deterioration and losses of bags.

Finally, the bags are left for 48 h into an oven at 80°C. Afterwards they are put into a desiccator prior to being weighed.

The validity of each replication is controlled according to DM before being partially grouped together. The nitrogen matter content is then determined by Kjeldahl method.

3.2.2. Results

Although the gas technique was slightly more efficient than the nylon bag in the identification of feeds with antinutritive factors, both techniques were poorly related to phenolic concentration

(Table 3). The reason for the lower efficiency of the nylon bag was problably due to the dilution of the antinutritive effects of phenolic compounds when incubated in the nylon bag in the rumen. There are two possible explanations for the lack of relationship between gas production and phenolics concentrations. The first is that the nature of tannins differs from one species to another. For example, one unit weight (1.0 g) of tannin from different sources showed different protein precipitating capacity which would suggest different biological response (Makkar *et al.*, 1993). Therefore it is very difficult to use a phenolic assay that can reflect different biological responses. Another less likely explanation, is that the concentration of substrate (i.e., 200 mg DM) to buffer:rumen liquor (30 ml) used according to Menke and Steingass (1988) is only 8% whereas in sheep it is about 2.5 g DM/30 ml rumen liquor. Thus the substrate concentration in the gas system might have been too low to show the maximum antinutritive effects and that more pronounced effects would have been measured if a higher concentration of the substrate had been used.

Table 3. Correlation coefficient (r) of the relationship between polyphenolic contents, fibre components, gas volume (ml/200 mg DM) or % DM degradation after 12, 24, 48, 72, 96 h and their (a+b) and (c) generated from the equation $p = a+b\ (1-e^{-ct})$ for the browse. Source: Khazaal and Orskov (1994)

Y variable	Correlation coefficient							
	TEPH	TETa	TECTa	TEPAa	NDF	ADF	Lignin	CP
NDF	-0.34	-0.03	-0.16	-0.12		0.81**	0.06	-0.34
ADF	-0.16	0.12	-0.15	-0.17			0.26	-0.33
CP	-0.04	-0.16	-0.33	-0.45			-0.07	
Lignin	0.27	0.04	0.24	0.35				
TEPH		0.77**	0.70**	0.56*				
TETa			0.55*	0.54*				
TACTa				0.85***				
Gas production after								
12 h	-0.22	-0.40	-0.34	-0.39	-0.29	-0.19	-0.07	0.53
24 h	-0.16	-0.41	-0.32	-0.38	-0.33	-0.30	-0.14	0.54
48 h	-0.14	-0.37	-0.29	-0.37	-0.37	-0.41	-0.28	0.48
(a+b)	-0.04	-0.29	-0.22	-0.39	-0.41	-0.53	-0.40	0.48
(c)	-0.23	-0.43	-0.31	-0.44	-0.05	0.11	0.21	0.61
DM degradation after								
24 h	-0.16	-0.51	-0.19	-0.31	-0.42	-0.48	-0.06	0.40
48 h	-0.10	-0.49	-0.10	-0.22	-0.48	-0.54	-0.05	0.37
(a+b)	-0.09	-0.52	-0.05	-0.15	-0.59	-0.70*	-0.09	0.39
(c)	-0.33	-0.42	-0.34	-0.42	-0.15	-0.18	-0.09	0.40

Number of samples: 40
Significance levels: *P<0.05; ** P<0.01; *** P<0.001

The failure of *in situ* DM degradation and the *in vitro* gas production to reflect the nutritive value of tannin rich feeds demonstrated the necessity for an alternative technique where phenolics-related antinutritive effects can be assessed more accurately.

Compared to other laboratory techniques, the gas production technique has been proved to be accurate in predicting animal performance (Blummel and Ørskov 1993; Khazaal *et al.*, 1993a, 1996) and while it was slightly inferior to nylon bag for determining nutritive value of feeds, it was suggested

as being more efficient than the nylon bag for determining the nutritive value of feeds containing antinutritive factors (Khazaal *et al.*, 1993b). There are also several other advantages that the gas production technique possesses which other techniques lack. Compared to the nylon bag, antinutritive effects would be more concentrated in the medium and more clearly observed. In comparison with other *in vitro* techniques, the gas production technique allows the monitoring of the kinetics of fermentation of the feed over long incubation periods without the need to use a large number of tubes to terminate treatment at different incubation periods. However, the greatest advantage the gas technique can offer is that unlike other laboratory techniques which measure residues after fermentation, it measures evolution of gas. Thus, if the negative effects of phenolics can be eliminated or neutralized by phenolics binding agents (PBAs) without interfering with the fermentation, the potential nutritive value of phenolic-rich feeds can be determined. Insoluble polyvinylpolypyrrolidone (IPVP) has been used for removing polyphenols from enzyme preparation, in tannin assays which are known to bind to phenolics without interfering with fermentation, much insight can be gained as to the nature of these antinutritional substances while at the same time improving fermentation conditions by their elimination.

At first it was necessary to investigate whether addition of PBAs interferes with fermentation. Results of studies by Khazaal *et al.* (1994) showed that IPVP can be added to a blank (rumen liquor:buffer) or a standard hay without affecting fermentation. Additionally, the incubation of 200 mg DM of the browse with equal amount of IPVP in the gas syringe increased the volume of gas produced in most of the browse compared to the untreated sample (Table 4). This increase as proportion of the untreated sample varied between species and with time. However, the tannins whether measured as TETa, TECTa, TEPAs or TOPAs were poorly related to the increase in gas production. At this stage, the lack of relationship between the increase in gas production and phenolics raised several questions in relation to the efficiency and quantity of IPVP:substrate and the medium conditions (e.g., pH) used (Khazaal and Ørskov, 1994; Khazaal *et al.*, 1996). One of the explanations of the results was that not all phenolic compounds that bind to IPVP were responsible for the increase in gas production or that IPVP is not binding to all antinutritive factors in the browse.

Regarding nitrogen matter degradability, results show (Table 5) that with the condition of washing with the stomacher method, sheep give the same classification regarding nitrogen value for the six forages studied (Tisserand and Faurie, 1994).

3.2.3. Discussion

Therefore further studies were carried out to investigate the reasons for the lack of relationship between improvement in gas production as a result of treatment with IPVP and concentrations of phenolics. This has been achieved by comparing the use of different molecular weights or quantities of different PBAs (e.g., polyethylene glycol PEG, soluble PVP) in the gas technique (Khazaal *et al.*, 1996).

It is possible to classify feeds according to nitrogen provided, using sheep instead of cows.

4. Conclusion

In order to accurately assess the nutritive value of Mediterranean forages and browse, it is necessary to use methods adapted to the peculiar characteristics of these forages.

After testing different prediction methods for intake and digestion on a large sample of Mediterranean forages, we have estimated the reproducibility and reliability of the methods used by chains of analyses involving all the teams participating in the project as well as 4 laboratories located in North Africa.

It is thus possible to propose a series of methods that we consider adapted and the use of which would allow results obtained in different laboratories to be comparable with each other.

Nevertheless regarding the dosification of tannin-type polyphenolics, it seems to be preferable, given the state of the art, to make an inventory of the possible methods while stressing those which are the most commonly used.

Table 4. Gas production (ml/200 mg DM) with or without 200 mg of IPVP after 24 or 48 h incubation. Source: Khazaal *et al.* (1993b); Khazaal and Orskov (1994); Khazaal *et al.* (1996)

Sample	Origin†/harvest	After 24 h			After 48 h		
		-IPVP	+IPVP	Increase (%)	-IPVP	+IPVP	Increase (%)
P. media	(GR) 5/91	34.8			42.0		
C. incanus	(GR) 6/91	14.9	16.8	12	18.8	22.1	17
C. incanus	(GR) 6/93	19.0	19.6	3	24.1	25.3	5
C. incanus	(GR) 9/93	16.4	17.6	7	20.4	22.1	8
A. andrachnoides	(GR) 4/92	19.1	21.8	14	26.5	30.3	14
A. andrachnae	(GR) 5/91	25.6	25.5	0	28.9	29.9	3
A. andrachnae	(GR) 9/91	17.0			25.3		
A. andrachnae	(GR) 6/93						
A. unedo	(GR) 6/91	28.0	28.2	1	31.4	32.3	3
A. unedo	(GR) 9/91	16.7	16.8	0	21.5	21.7	1
A. unedo	(GR) 9/93						
C. duinensis	(GR) 6/91	21.1	22.7	8	26.3	29.0	10
C. duinensis	(GR) 9/91	21.3	23.8	11	27.7	30.6	10
C. duinensis	(GR) 9/92	20.0	21.9	9	25.0	27.5	10
Q. coccifera	(GR) 6/91	17.2	21.0	22	25.0	28.4	14
Q. coccifera	(GR) 9/91	15.5	16.8	9	21.0	22.3	6
F. ornus	(GR) 6/91	27.7	28.9	5	34.8	36.4	4
F. ornus	(GR) 9/91	27.0	30.8	14	36.2	38.3	6
F. ornus	(GR) 9/92	25.4	28.2	11	36.6	38.6	6
R. pseudoacacia (GL)††	(GR) 9/93	26.5	26.3	0	30.2	30.4	0
R. pseudoacacia (UL)††	(GR) 9/93	24.0	23.9	0	28.3	28.4	0
R. pseudoacacia (UB)††	(GR) 9/93	22.1	21.5	0	26.5	25.9	0
Q. pubescens	(GR) 6/93	11.0			16.0		
Q. pubescens	(GR) 7/93	15.6			19.5		
Q. pubescens	(GR) 9/93	15.7			19.6		
J. oxycedrus	(GR) 6/93	24.2			30.0		
J. oxycedrus	(GR) 9/93	19.0			22.0		
A. campestris	(SP) 7/91	25.1	29.5	17	30.5	34.9	14
Q. rotundifolia	(SP) 11/91	15.2	16.5	9	20.6	22.3	8
T. polium	(SP) 7/91	28.6	29.6	4	33.0	33.5	1
S. chamaecyparissus	(SP) 7/91	25.6	24.4	-4	32.3	31.1	-4
H. stoechas	(SP) 7/91	25.4	31.8	25	30.5	38.1	25
R. officinalis	(SP) 11/91	25.8	28.6	11	30.0	32.0	7
A. decorticans	(SP) 11/91	35.1	35.5	1	38.7	39.3	2
Q. coccifera	(FR) 6/91	14.8	18.5	25	21.3	26.2	23
A. cyanophylla	(TU)	20.8			24.4		
A. cyanophylla	(TU)	15.2			20.0		
A. campestris	(TU)	24.3			28.8		
A. herba alta	(TU)	25.8			29.5		
A. halimus	(TU)	30.3			32.0		
C. siliqua	(TU)	25.5			33.2		
C. libanotus	(TU)	31.5			38.5		
C. salvigolus	(TU)	17.1			24.9		
G. alypum	(TU)	21.6			26.4		
J. phoenicea	(TU)	24.3			29.7		
O. ficus indica	(TU)	45.9			54.8		
P. angustifolia	(TU)	22.4			31.5		
P. lentiscus	(TU)	12.2			15.3		
R. officinalis	(TU)	14.7			14.6		

†GR: Greece; SP: Spain; FR: France; TU: Tunisia
††GL: Grazed leaves; UL: Ungrazed leaves; UB: Ungrazed braches

Table 5. Results of nitrogen compound degradability in cows and sheep according to type of wash

Forages	Time	N eliminated			
		Classical wash		Stomacher wash	
		Cows	Sheep	Cows	Sheep
Common honey locust	2	63.19	46.78	66.27	57.5
1 mm	4	77.08	62.13	66.66	66.27
	8	81.98	49.82	83.92	74.89
	16	87.3	82.34	88.82	86.72
	24	87.94	84.63	88.89	89.75
	48	89.67	86.39	90.8	90.35
Fraxinus ornus	2	18.24	19.07	17.75	28.78
1 mm	4	24.44	16.6	22.86	24.47
	8	39.42	31.69	53.05	41.63
	16	76.74	68.18	80.13	63.59
	24	80.32	70.77	83.62	77.99
	48	82.27	72.45	85.21	82.3
Strawberry madrone	2	9.05	14.43	10.73	14.03
5 mm	4	8.93	16.97	14.51	19.35
	8	---	14.18	15.7	17.28
	16	17.39	12.97	31.69	31.55
	24	6.27	5.45	55	32.56
	48	58.16	26.99	69.84	59.04
Quercus coccifera	2	28.64	32.4	35.78	37.55
1 mm	4	28.23	31.82	35.86	39.32
	8	30.37	25.75	39.04	37.16
	16	35.84	30.54	44.64	43.03
	24	32.57	29.54	---	46.53
	48	34.66	30.38	40.39	45.24
Quercus pubescens	2	18.9	22.03	27.46	29.02
1 mm	4	16.23	23.09	27.41	25.07
	8	17.44	22.4	34.95	28.95
	16	34.62	28.5	51.96	38.6
	24	35.21	35.23	59.48	45.74
	48	46.1	41.5	64.69	59.56
Durum wheat straw	2	10.36	23.82	39.56	47.11
5 mm	4	10.55	23.37	44.61	46.56
	8	21.31	15.53	45.47	49.32
	16	21.1	21.17	54.4	47.92
	24	22.82	9.45	52.98	43.73
	48	18.56	15.98	65.43	50.04

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Second Part

Recommended Methods

Technique of *in vivo* determination of intake and digestibility of forages

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It is important to stress that the following guidelines are essential but also allow for some flexibility of common sense in relation to the conditions under which the trial is carried out.

1. Intake

Time: the minimum time required for determining feed intake is 4 weeks. The mean of the last two weeks can be used.

Animals: the animals must be weighed each week and the mean of the last 2 weights can be used to express intake relative to weight or metabolic weight. The live weight of the animals must always be reported and if possible the rumen must have reached mature proportions. The body condition and if known the previous nutrition must be commented on. The animals must be free from intestinal parasites.

The feed: make sure that the conditions of the feed do not change during the trial. If possible in small ruminant work, make up the feeds for the whole experiment at one time. Additionally, the following points should be taken into consideration.

1.1. Quality

If the roughage in question contains less than 2% crude protein for every 10% estimated digestibility (approximately) then a flat rate of high protein cake should be given to the animal twice daily to make up the deficit. Soya, cottonseed or sunflower, etc. can be used. We must be sure that intake is not limited by deficiencies in the diet. If a few diets are tested in the same design, the same flat rate should be used for all, based on the diet with the lowest CP.

If the roughages are very poor keep an eye on the condition of the animals. It may be necessary to feed a small amount of a high quality fibrous supplement to ensure that the animals do not lose too much weight.

If animals are confined they should have access to a mineral lick. This is particularly important for poor quality roughages.

The environment in which the intake is determined must be described e.g., average temperature and temperature fluctuations.

1.2. Physical form

If a series of roughages is to be tested they should either all be fed in the long form or chopped.

For sheep and goats, chopping is useful and makes experimental diets easier to weigh out. Suggest long roughages for cattle. Most silages are now chopped but this can also be used.

1.3. Dry matter content

Ideally, dry matter of the feed offered should be determined every time the feed is given to the animal. This is necessary when silages or fresh green material are fed to the animals. Silages must if possible be corrected for volatile compounds e.g., acetic lactic NH_3. Time can be saved by weighing out dry roughages for a week at a time.

1.4. Number of observations

At least 6 observations for each feed are needed. If possible more, but 6 is minimum and preferably the same age, breed and sex. Change over design can be used for intake studies. It would be desirable also if at least 6 feed but possibly many more could be done at each station.

Refusals: the animal should be allowed to refuse about 10-15% for cattle and 20-25% for sheep and goats for shrubs and browse; other conditions need to be introduced such as the woody material not eaten and could be much more than 20-25%. The dry matter (DM) content of the refusals must be measured. For sheep and goats, the degradability measurements must be done on the refusals. In the case of shrub feeding it is necessary to feed maybe 3 times more than the animals will consume as often only the leaves are consumed. It is especially important that the dry matter concentrations and weight, both the feed offered and refused are measured.

Feeding frequency: the feed must be introduced twice daily, but the refusals need only be recorded once daily.

2. Digestibility

In vivo digestibility is essentially measured for 2 purposes: (i) for some standard feed evaluation methods *in vivo* digestibility must be measured at maintenance; and (ii) digestibility measured as part of *ad lib* consumption trials.

For both purposes faeces should be collected for at least 7 days. For purpose (i) a preliminary period for adoption of 7 days is also required. For purpose (ii) it should be made after the feed intake trial. Faeces can be collected either in faecal collection bags fitted to a harness or in metabolism crates or stalls allowing for collection of faeces and separation of urine and faeces.

Faeces can be stored at 0°C for the collection period after which it must be mixed and subsampled for appropriate analysis. Generally faeces should be dried at 60°C for approximately 48 hours feed refusal if any must be recorded and analysed for each individual animal. If urine is collected it must be collected in dilute sulphuric acid to ensure that Pn is less than 3 when urine is collected.

The environment, e.g., temperature at which the digestibility trial was carried out must be recorded. It is desirable that digestibility trials are carried out in-doors or under a roof.

Design: for purpose (i) change over designs can be used if several feeds are involved; for purpose (ii) the digestibility trial is simply done at the end of each period of feed intake measurements and the design depends on the design for the intake trial.

Technique of *in vivo* determination of intake and digestibility of browse

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1. Introduction

There are numerous methods for determining the nutritive value of shrubby forages, as reviewed by Nastis and Meuret (1987). The use of a specific method depends on the objectives and the available resources. Undoubtedly, the *in vivo* feeding trial procedure is the one that most accurately determines the nutritive value of a given feed, since it measures the actual amount of feed ingested and digested. There are general guidelines that should be taken into consideration when planning and conducting feeding trials. The most important are the following.

2. Experimental set-up

Digestion trials have to be conducted indoors, so that environmental variations which can affect the intake and, indirectly, the digestibility of the feed, are reduced. Experimental outfits with a controlled "thermoneutral" temperature are preferred. The animals are confined in metabolism cages, to which an arrangement of sieves is attached to separate faeces and urine. The cages have to be wide enough to accommodate the animals, which (especially goats) should be chained so that they can move freely without being able to jump out.

3. Animals

3.1. Number of animals

It is important to have enough animals in a digestion trial. Most experiments use 4-10 animals per treatment depending on the available resources. The higher the number of animals the more accurate and reliable the results.

3.2. Selection of animals

In order to minimize variability, mature animals of similar body weight and age of the same sex and breed are selected. If females are used, they should not be pregnant, to avoid unnecessary stress and variability due to differing physiological requirements.

Animals that show signs of behavioural stress (e.g., nervousness, low intake, weight loss) due to confinement in metabolism cages, should be replaced with similar calm ones before proceeding with the digestion trial. Results obtained from aberrant animals usually deviate greatly from the norm. It is important also to make sure that the animals are healthy and free from internal parasites. To eradicate internal parasites, each animal is dosed with albendazole 600, 3.8 mg/kg B.W before the experimentation.

4. Feeding procedure

The experimental animals should be given enough time to become accustomed to living in the metabolic cages and the test feed. This is normally achieved within 7-10 days (preliminary period), during which the animal microflora adapts to the test feed.

4.1. Quality

If the crude protein content of the test feed is less than 2% for every 10% of estimated digestibility units, a protein supplement (such as soya or cottonseed) must be used to make up the deficit. Animals should also have continuous access to mineral licks and clean water throughout both the preliminary and the test period.

4.2. Quantity

It is important to ensure that the amount of feed to be used is sufficient to cover the whole duration of the experiment. The food used for the trial should be as homogeneous as possible. With conventional feeds, two meals per day are offered and the amount of feed offered is approximately 10% more than the maximum predetermined voluntary intake. In the case of shrub feeding, the feed is offered continuously throughout the day. In order to simulate natural grazing where feed selection exists, the feed should be offered as branches in quantities of about 3 times the estimated daily intake. To assess the dry matter intake, the percentage of weight lost through evaporation in the course of the day must be calculated by setting aside a batch of feed similar to those offered to the animals and weighing it at the start and at the end of daily feeding.

Intake is calculated from the difference between the feed offered and the feed rejected, taking into account evaporation loss.

5. Sample collection

The cages should be cleaned thoroughly before the collection period. Collection normally starts after the 7-10 day preliminary adjustment period.

Faeces and urine are collected at the same time once a day and stored composted in separate containers in a refrigerator. In order to avoid loss of ammonia from urine due to evaporation, 50 ml of 25% H_2SO_4 is added to the urine collectors daily. Sub-samples from the batch of branches set aside are taken by hand plucking in imitation of the browsing behaviour of the animals.

Two homogeneous sub-samples are obtained. One sub-sample of feed and of faeces is stored in a deep freeze for possible later use. Part of the other is oven-dried at 105°C to determine the DM content. A 10 ml sample of urine is weighed and used to determine the weight of the total urine output. The rest of the urine sample is freeze-dried and used for chemical analyses.

Up to 10 days of total collection is sufficient to assess a feed accurately. The animals are weighed two days before collection starts and at the end of the collection period. Before weighing they are deprived of feed and water for 12 hours. Animal body scoring has to be monitored through the experimentation.

6. Results and calculations

When the experiment is over, the total amount of daily feed intake (kg DM/$BW^{0.75}$) and daily excretion of faeces (kg DM) and urine (litres/day) are calculated.

Feed, faeces and urine samples are analysed for nitrogen and energy content. Freeze-dried urine samples can be burned in a bomb calorimeter by placing samples in plastic cups or impregnated cellulose. Alternative energy estimates can be made by the use of existing equations.

The digestibility of a feed (DM or OM) or any constituent of the feed is calculated as follows:

$$\text{Digestibility \%} = \frac{\text{Feed intake - Faeces}}{\text{Feed intake}} \%$$

Nitrogen and energy balance can be calculated in the same way:

Nitrogen balance = (% N in dry feed x daily dry wt of feed) - (% N in dry faeces x daily dry wt of faeces) - Daily urine N.

Energy balance = (Cal content of feed x daily wt of feed) - (Cal content of faeces x daily wt of faeces) - Daily energy content of urine.

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Technique of *in vitro* digestibility: The two stage method

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1. Principle

Feedstuff samples are incubated with rumen liquor for 48 hours, followed by an enzyme hydrolysis.

The samples are filtered and washed. Each residue is dried at constant weight and incinerated for the calculation of its Dry Matter and Organic Matter.

The difference between incubated Dry Matter and/or Organic Matter and the amount recovered after incubation and washing reveals the digesta.

2. Reagents

All reagents have to be of analytical quality. The water has to be distilled or equivalent in purity.

2.1. Rumen buffer solution (McDougall's synthetic saliva)

2.1.1. Macromineral solution

- Sodium Hydrogen Carbonate (Na HCO_3): 9.8 g/l H_2O g/l H_2O
- di-Sodium Hydrogen Orthophosphate 2 - hydrate (Na_2 HPO_4 $2H_2$ O): 4.9 g/l H_2O
- Potassium Chloride (K Cl): 0.57 g/l H_2O
- Sodium Chloride (Na Cl): 0.47 g/l H_2O
- Magnesium Sulphate (Mg SO_4 $7H_2$ O): 0.12 g/l H_2O
- Calcium Chloride (Ca Cl_2): 0.04 g/l H_2O

With ±0.1 mg precision, weigh the quantities mentioned for each salt.

In a small amount of water, individually dissolve the carbonate and the phosphate on one hand and the chlorides and the sulphate on the other. Add both solutions and fill with distilled water up to 1000 ml. The solution will present a cloudy aspect because of the Calcium Chloride.

2.1.2. Micromineral solution

- Iron Sulphate (Fe SO_4 $7H_2O$): 3.68 g/l H_2O
- Manganese Sulphate (Mn SO_4 H_2O): 1.90 g/l H_2O
- Zinc Sulphate (Zn SO_4 $7H_2O$): 0.44 g/l H_2O
- Cobalt Chloride ($CoCl_2$ $7H_2O$): 0.12 g/l H_2O
- Copper Sulphate (Cu SO_4 $5H_2O$): 0.098 g/l H_2O
- Ammonium molybdate ($M0_7$ $(NH_4)_6$ O_{24} $4H_2O$): 0.0174 g/l H_2O

On the day before incubation and for each 5 l of the macromineral solution, add 50 ml of the micromineral solution, stir and place in a refrigerator.

Saturate the buffer solution with carbon dioxide during approximately 15 minutes and later measure the pH which should be near neutrality (±0.1 pH units). Adjust if necessary with NaOh or HCl.

This synthetic saliva solution has to be used at 38-39°C.

2.2. Solution of hydrochloric acid 1 N

2.3. Solution of hydrochloric acid 5 N

2.4. Pepsin solution in hydrochloric acid

With ±0.1 mg precision, weigh 2 g of pepsin (1:10000) and pour into a 1000 ml flask containing some distilled water prewarmed to 39°C. Completely dissolve the pepsin and add 100 ml HCl 1N (2.2). Fill up to the total volume and homogenize.

This solution has to be prepared freshly for each set of trials and immediately before utilization.

3. Apparatus

Common laboratory material and namely the following:

(i) System for collection and preparation of rumen content (Fig. 1)

- 2000 ml Kitassato fitted with a perforated rubber stopper and a polyethylene tube
- Rubber manual pump or vacuum pump
- Porcelain funnel with filtering plate
- 1000 or 2000 ml filtering bottle with hose connection
- Vacuum system
- Gauze
- Thermos flask

(ii) Magnetic stirrer

(iii) Automatic volume dispenser of 50 ml

(iv) Thermostatized water bath set to 39-40°C, with stopper

(v) Oven set to 103 ± 2°C

(vi) Desiccator with dehydrating agent

(vii) CO_2 container

(viii) Glass tubes with rubber stopper fitted with Bunsen valve (Fig. 2)

(ix) 50 ml crucibles with G_2 filtering plate

(x) Muffle furnace set to 550 ± 20°C

4. Donor animals

Three rams or cows fitted with a rumen cannula, fed a good quality basic diet of hay at maintenance level. If necessary, they may be given a protein concentrate as supplement (for instance, soybean cake) so that the diet has approximately 12-13 of CP % DM.

The hay is chopped into ±5 cm particles and the total diet is given as a single meal/days. The collection of rumen content takes place before the meal.

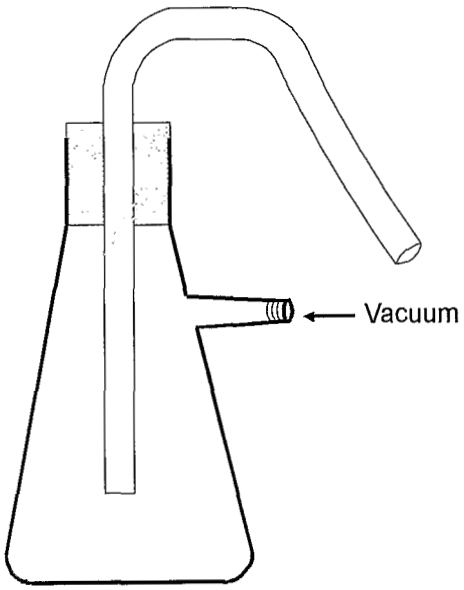

Fig. 1. System for rumen contents collection.

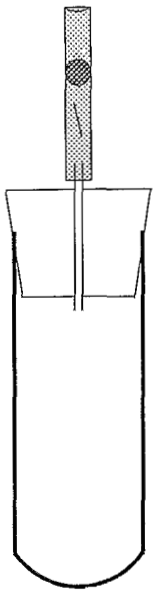

Fig. 2. Centrifuge tube fitted with rubber stopper and busen valve.

5. Procedure

The determination of dry matter and ashes in the samples has to be carried out in the same manner.

5.1. Preparation of the sample

The sample has to be dried at 65°C in a ventilated oven and crushed so that it will pass a 1 mm screen.

Then with a precision of ±0.1 mg, weigh 0.5 g of the sample to an incubation tube. Two replicates of each sample must be weighed.

5.2. Collection of the rumen content

All of the material has to be prewarmed to 38-39°C. Collect the rumen content of the 3 donors before the meal by means of the system mentioned in 3(i).

Filter through 4 layers of gauze with the help of the porcelain funnel with porous plate into a 1000 or 2000 ml filtering bottle, under vacuum.

Quickly transfer the filtrate to a thermos flask (avoiding air contact) until incubation.

5.3. Incubation

5.3.1. First stage

Mix 1 part of the rumen fluid with 4 parts of the synthetic saliva in a flask positioned in a water bath set at 39°C and stir by a magnetic stirrer. Continuously introduce CO_2 in the flask in order to maintain the conditions of anaerobiosis of the medium.

Adapt an automatic volume dispenser to the flask and give 50 ml of the rumen fluid - synthetic saliva mixture into each tube containing the sample, prewarmed to 39-40°C and positioned in a bath thermostatized at this temperature.

Simultaneously introduce CO_2 in each tube and immediately stopper the tubes fitted with the Bunsen valve (Fig. 2).

5.3.2. Second stage

This stage starts after 48 hours of incubation.

Add 2 ml of hydrochloric acid 5 N in order for tubes to have a pH of 1.2-1.3.

Then add to each tube 50 ml of the pepsin solution (2.4) prewarmed to 40°C with the help of the automatic dispenser. Stopper the tubes and position them in a thermostatized bath during 48 additional hours, stirring manually occasionally.

After the incubation period, open the tubes, wash the stoppers and filtrate with the help of vacuum through previously tared 50 ml crucibles with a G_2 filtering plate. Wash the tubes with warm distilled water to remove any residue.

Dry the crucibles during the night in an oven at 103°C and weigh. Incinerate at 550°C during at least 4 hours, or overnight and weigh the residue after careful cooling in a desiccator.

5.4. Trial using standard samples

Simultaneously carry out a trial with at least 4 standard samples which have digestibilities that reach those of the trial samples. Follow the technique described in 5.2.

Four standards are suggested: 1 hay, 1 straw, 1 maize silage and 1 cereal grain.

In routine, these standards are enough to cover the digestibility of samples. When necessary a by-product with an *in vitro* DM digestibility <25% is also introduced.

5.5. Blank test

Simultaneously carry out a blank test 4 times according to the technique described in 5.2 and remove the sample.

6. Results

6.1. Calculation

The *in vitro* digestibility of dry matter (DMD) and organic matter (OMD) as % is calculated as follows:

$$\text{DMD (\%)} = 100 \times \frac{\text{(corrected DM) - (residue DM)}}{\text{incubated DM}}$$

$$\text{OMD (\%)} = 100 \times \frac{\text{(corrected OM) - (residue OM)}}{\text{incubated OM}}$$

where: corrected DM = initially incubated DM + DM from the blank test; and corrected OM = initially incubated OM + OM from the blank test

Consider as the result the arithmetic average of the parallel determinations.

6.2. Correction of results

Draw a curve of reference (regression line) with the digestibilities obtained for the samples as ordinate and the corresponding mean digestibilities as abscissa.

Apply the reference curve to the results obtained for samples in 6.1 and estimate the corrected *in vitro* digestibilities.

6.3. Presentation of results

The results are rounded off to decimals.

6.4. Repeatability

The difference between the results of the two determinations carried out by the same operator during successive weeks must not exceed 3% in absolute value.

Note: Contrarily to *in vivo* values, the OM *in vitro* digestibility is inferior to that of DM due to solubilization phenomena of certain minerals in the acid medium which disappear during filtration, thus decreasing the residual ash content.

7. Final comments

In vivo techniques are slow, they require great quantities of feed and adequate installations. At present, it is necessary to predict the digestibility of a large number of feeds in the shortest time possible and with small quantities. The development of *in vitro* techniques has been the answer to these problems and has given us the possibility to estimate the *in vivo* values with considerable rigour.

The *in vitro* digestion trials only reflect the potential nutritive value of the feed being studied. Care should be taken not to use this method erroneously in the prediction of certain nutritive parameters (example: intake).

Depending on the donor animals, the type of sample and the technique used, *in vitro* results may be equal, greater or lesser than the *in vivo* results. Therefore, a careful control of all methods used is necessary. Normally, samples that have a digestibility between 60 and 70% obtain *in vitro* results that are higher than *in vivo* results (Serrano, 1979). However, it is common to have values that are 1 or 2 units below the *in vivo* values.

The interpretation and application of results is also very important. Many forage species form such a uniform pattern in their relation between *in vitro* and *in vivo* values, that they may be grouped together. However, for the study of forages, advice is given for the utilization of *in vitro* methods with a previous establishment of regression equations which have a validity that depends on some of the *in vivo* trials being carried out simultaneously.

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The nylon bag technique for the measurement *in situ* of rumen degradability

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1. Introduction

The rumen degradability of a feedstuff is the extent of its digestion in the reticulo-rumen and it has been proposed as a method for predicting animal performance; the *in situ* nylon bag technique is the most commonly used method for estimating degradability. The application of this technique leads to the characterization of feeds in terms of an immediately soluble fraction ("A") and a potentially degradable fraction ("B") which is fermented at a constant rate ("c"). The technique can be applied to study the fermentation characteristics of the dry and organic matter content, fibre fractions and, particularly for concentrates, nitrogen.

The method is widely used and much information concerning conventional feedstuffs and by-products has been published in the literature, although data on Mediterranean feeds is still restricted to a limited number of browse and other forage crops and for these Mediterranean feeds the evidence for a relationship between *in situ* data and animal performance is inconclusive.

The characterization of feeds in terms of fermentation characteristics would be a rapid and relatively inexpensive method of predicting their nutritive value. In comparison with feeding trials, a lower number of animals is required and within one week, the typical collection period for a digestibility trial, several feeds can be evaluated with the same animals. Moreover, it has often been reported that *in situ* rumen degradability is not subject to a significant effect of species, and this allows the use of cattle, sheep or goats, depending on local availability.

The method is very easy in principle and requires only a short training period. The equipment required is simple and found in all nutrition laboratories - a balance capable of weighing to at least two decimal places, an oven and a hammer mill.

2. Animals

Dependent upon local availability and experimental aims. Preference given to cattle due to the number of incubatable bags, but goats and sheep are acceptable if the cost of maintaining cattle is too high.

At least 3 animals, preferably 4 or more.

Animals to be maintained by trained staff in accordance with local animal welfare regulations. Special attention must be given to the animals' feet.

The fistula and cannula should be cleaned regularly. The fistula should be examined for lesions and the cannula for damage.

3. Basal diet

Adequate for maintenance or slightly above. Preference to be given to a hay and concentrate diet (3:1 DM basis), but it is more important that adequate energy and protein are available to ensure satisfactory rumen microbial activity. A guideline of 2% crude protein per 10% dietary DM digestibility

is recommended as a minimum. Diet to be adequate in minerals, vitamins and trace elements (especially sulphur).

Dry matter intake about 1.0% of liveweight.

4. Bags

Preferably round-bottomed nylon bags (with heat sealing or nylon stitching) with pore size in the range 40-60 μ (to be specified in report). Always use the same sized bags within any one experiment.

Size: not important but sample weight: free surface area = 12-18 mg/cm^2.

Tied to a weighted support system to ensure maximum available surface area is exposed to the rumen environment and that the bags are held down in the mass of rumen solids and liquor.

5. Sample preparation

Samples to be incubated in a state as close to that eaten by the animal as reasonably possible.

Dry forages to be milled through a 2 mm (minimum) or (preferably) 4 mm screen (state in report).

Wet forages: store frozen if necessary; cut to 1-2 cm long or mince frozen through a 5 mm screen.

Concentrate feeds: always use a screen larger than 1 mm.

6. Incubation times

Adequate to define degradability curve. For forages, at least 6 times are recommended, for example 8, 16, 24, 48, 72 and 96 h, and 5 times for concentrates, e.g., 2, 6, 10, 24 and 48 h. The essential requirement is to adequately describe the asymptote (i.e., $a + b \leq 100$, see below). The difference in degradability between the penultimate and last incubation time should be less than 10% of the penultimate value; i.e., if the penultimate value is 40% the last value should be less than 44%.

A "zero time" incubation or solubility ("A") measurement should be made by preparing at least two additional bags per feed and washing them (as below) without incubating them in the rumen.

7. Washing procedure

Bags to be removed from animals and dipped in cold water to remove excess rumen solids.

If possible, bags to be washed in cold rinse cycle of a washing machine followed by a short, low-speed spin.

If a washing machine is not available, bags should be washed by the same member of staff under cold running water, taking care not to squeeze the bags and using the same procedure each time.

8. Drying procedure

Bags to be dried in a ventilated oven at a maximum temperature of 60-65°C for at least 48 hours. Bags should be placed on absorbent paper or similar material to prevent them touching the hot metal surfaces. If possible, bags should be transferred to a desiccator prior to weighing, otherwise they can be left to re-equilibrate to atmospheric humidity (overnight) and then the weights corrected for residual humidity.

9. Interpretation of the data

Examine the data for outlying values. Plot the data to see if there is any evidence of lag phases.

If no lag phase, interpolate with:

$$p = a + b \cdot (1-e^{-ct}) \qquad \text{[equation 1]}$$

where "p" is the observed disappearance of material from the bags, "a" is the intercept of the degradability curve on the y-axis (often referred to as the rapidly soluble fraction), "b" is the fraction degradable with time and "c" the constant rate of degradation. The zero time disappearance values should not be included in the interpolation process.

The effective degradability, Dg, which combines the rumen disappearance of matter from the bags with the outflow rate from the rumen (k, expressed as a decimal fraction per hour) can be calculated from:

$$Dg = a + [(b \cdot c) / (c + k)] \qquad \text{[equation 2]}$$

where a, b and c are the coefficients from equation 1 and k is the rumen outflow rate (Ørskov and McDonald, 1979).

If there is a lag phase, begin by interpolating as shown in equation 1 (again excluding the zero time values). The effective degradability can then be calculated from:

$$Dg = A + (bc) / [(c + k) \cdot e^{(-(c+k) \cdot L)}] \qquad \text{[equation 3]}$$

where A is the observed washing loss, b and c are the interpolated coefficients as in equation 1;

B = (a + b) - A, where B is the degradable fraction

L = (1/c). 1n (b/B) where "L" is the lag phase in hours.

Programs are available to calculate degradation, the lag phase and the effective degradability of feeds. These can be obtained from Dr. E.R. Ørskov, International Feed Resource Unit, Rowett Research Institute, Bucksburn, Aberdeen, AB2 9SB, Scotland, UK.

The use of these modified equations has been discussed by Ørskov and Ryle (1990) and McDonald (1981).

10. Presentation of the results

Give the degradability coefficients a, b, c, A, B as appropriate. Effective degradability can thus be calculated by the reader for a given rumen outflow rate.

In calculating protein degradability, if it is possible and considered necessary to correct for microbial contamination, this should only be done for forage samples.

11. Limitations

Essentially for DM and protein, but has been extended to fibre fractions. N degradability of forages should not be evaluated, unless corrected for bacterial nitrogen contamination. For protein supplements, a correction for microbial contamination is not required. Ring tests on reference feeds have often demonstrated a low reproducibility of the technique and the application of a standard protocol is not sufficient to increase the accuracy of the measurement. Collaborative studies among laboratories, together with the use of reference feeds, is recommended when degradability studies are to be carried out.

Some Mediterranean feeds are known to contain very high concentrations of tannins which are indigestible and may interfere with the degradation and digestion process. As a portion of these compounds will be soluble in water, some caution should be exercised in the interpretation and utilization of the "A" value and the effective degradability calculated for such feeds as there will be a tendency to overestimate the degradable, and thus digestible, fraction. However, this effect will be diluted if bags containing only a few grammes of test material are incubated in the rumen of an animal consuming a basal diet of hay and/or dried grass and concentrates. The antinutritional effect can be demonstrated in closed *in vitro* systems.

Fistulated animals are required and this may represent a constraint in some western countries.

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Estimation of phenolic compounds content

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Phenolic compounds are a complex group of substances and include insoluble molecules (like lignins) and soluble molecules (like tannins). Both interfere with the nutritive value of forages. Quantitation helps to understand and anticipate their effects in diets.

1. Lignin determination

Chemical methods for lignin determination (Giger, 1985) can be divided in:

(i) Lignin quantitation. The most used method is based on van Soest and Wine's method (1967): cell wall compounds are quantified by differential weighings after successive hydrolyses.

(ii) Qualitative methods which give information about composition of lignins.

1.1. Lignin quantitation: van Soest and Wine's method modified by Giger (1987)

This method was used for a ring-test of the EEC project 'Evaluation des ressources fourragères locales dans les régions méditerranéennes' No. 8001-CT90-0022.

1.1.1. Sample conditioning

(i) Dried samples.

(ii) Milling 5 or 1 mm according to the sample.

1.1.2. Apparatus and reagents

(i) Fibertec system corresponding to a hot extraction unit and, if possible, a cool extraction unit.

(ii) Crucibles No. 2.

(iii) Drying oven and ash oven.

(iv) Neutral detergent solution (NDS):

- Weigh the different following chemicals:
  . di-sodium hydrogen phosphate anhydrous (Na_2HPO_4): 4.56 g
  . titriplex III ($C_{10}H_{14}Na_2O_8$, $2H_2O$): 18.61 g
  . di-sodium tetraboratedecahydrate ($Na_2B_4O_7$, $10H_2O$): 6.81 g
  . dodecyl sulphate sodium salt ($C_{12}H_{25}NaO_4S$): 30 g
- Add about 800 ml tepid distilled water and mix (magnetic stirrer)
- After dissolution and cooling adjust to 1 l with distilled water (be careful with the foam)

(v) Acid detergent solution (ADS):

- Weigh 20 g N-cetyl-N,N,N trimethylammonium bromid (CTAB) $C_{19}H_{42}BrN$
- Add about 800 ml distilled water and 28.8 ml H_2SO_4 (96%) and mix (magnetic stirrer)
- After dissolution and cooling adjust to 1 l with distilled water

(vi) Acid detergent lignin (ADL) solution:

- H_2SO_4 72%

1.1.3. Method

Remarks: make always 2 determinations each sample. All weights are obtained after cooling crucibles in desiccator.

(i) Weigh clean and dry crucible → P0.

(ii) Weigh crucible + 2 g sample - dry (crucible + sample) at 100°C for 48 h.

(iii) Weigh (crucible + sample) for DM (dry matter) determination → P1.

Remark: for lipid-rich samples (>10% DM) use modified Folch's method. Eliminate lipids 3 times:
- First: add 15-20 ml solution chloroform/methanol (2:1; v/v) to 2 g dried sample in crucible at 4°C for 1 h - filter
- Second: idem
- Third: idem but extract during a whole night at 4°C - filter and wash with boiling water - transfer (crucible + sample) directly to the boiling position and go on with NDF obtention

(iv) NDF obtention:

- Transfer crucibles to the boiling positions into the hot extraction unit
- Add 100 ml preheated NDS in each column (see Fig. 1)
- Adjust heat and boil for 60 min
- Filter NDS and wash at least 5 times each sample with boiling water to eliminate NDS. Each time use about 30 ml and suck as dry as possible. If necessary remove sediment from the filter surface by applying reversed pressure
- Dry (crucible + sample) at 100°C for 48 h - weigh → P2

Remark: for starch-rich samples (>4% DM):
- 2 g sample
- + 100 ml boiling NDS - boil for 30 min - filter NDS
- Add 100 ml cool NDS + 4 ml amylase solution (2%) - boil for 30 min
- Filter NDS, wash and dry as above → P2

(v) ADF obtention:

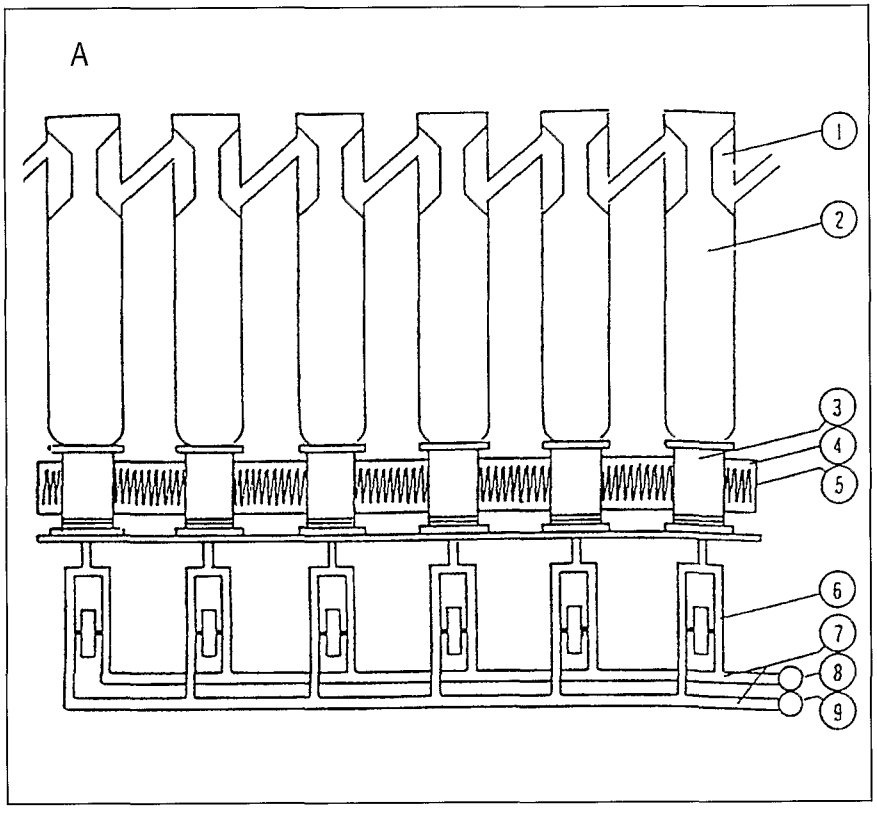
- Wet NDF residues in crucible with distilled water to break lumps
- Transfer directly crucibles to the hot positions - filter water
- Add 100 ml preheated ADS in each column - adjust heat and boil for 60 min - filter and wash as above
- Dry (crucible + ADF residues) at 100°C for 48 h and weigh → P3

(vi) ADL obtention:

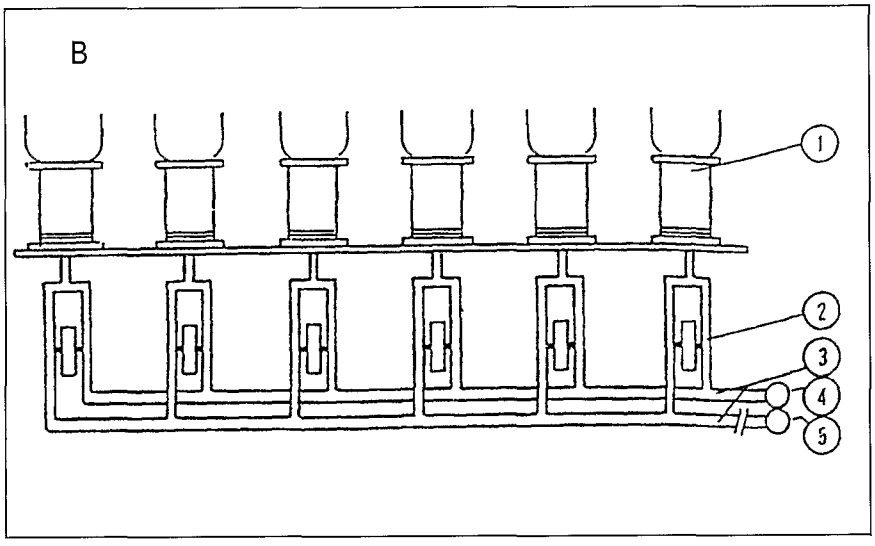
- Transfer (crucible + ADF residues) to the positions or in plastic becher
- Add 10-20 ml H_2SO_4 72% and break lumps
- Wait 3 h at room temperature then wash and dry as above - weigh → P4

(vii) Ash obtention:

- Ash the sample in the crucible at 570°C for 5 h - weigh → P5



1. Condensers
2. Columns
3. Crucibles
4. Boiling heater
5. Regulator for boiling heater
6. Control valves
7. Glass manifolds
8. Water suction pump
9. Pressure pump



1. Crucibles
2. Control valves
3. Glass manifolds
4. Water suction pump
5. Pressure pump (in Hot Extraction Unit)

Fig. 1. Hot Extraction Unit. A: general view; B: detail.

1.1.4. Expression of results

(i) Express NDF and ADF to the DM of the sample:

- % NDF = (P2-P0)/(P1-P0)
- % ADF = (P3-P0)/(P1-P0)

(ii) Express ADL to the DM or OM (organic matter) of the sample:

- % ADL = (P4-P0)/(P1-P0) or % ADL = (P4-P5)/(P1-P5)

1.1.5. Remarks about this method

(i) Advantages:

- Reproducible
- Easier than Weende cellulose method
- Most commonly used for cell wall determination

(ii) Disadvantages:

- Cost of the extraction unit
- Filtering problem with some substrates
- We can only approximate values for the different parts of the cell wall because Maillard complexes, condensed tannins, some cell wall proteins and cuticular waxes can interfere (Giger, 1985). Approximations are: lignin ~ (P4-P5), cellulose ~ (P3-P4), hemicellulose ~ (P2-P3)

1.2. Lignin qualitation using alkaline nitrobenzene oxidation (Reeves, 1986; Lapierre *et al.,* 1988)

This method is proposed by B. Stefanon, Dipartimento di Scienze della Produzione Animale, Università degli studi di Udine, 33010 Pagnacco, Italy.

1.2.1. Reagents

(i) Neutral detergent solution according to Goering and van Soest (1970).

(ii) Nitrobenzene.

(iii) Petroleum ether.

(iv) Concentrated sulphuric acid.

(v) Methanol.

(vi) Pure phenolic monomers.

1.2.2. Materials and apparatus

(i) Steel screw-capped tubes lined with teflon.

(ii) Oil bath.

(iii) Single pump high pressure HPLC with loop injector.

(iv) Variable wavelength UV detector.

(v) Integrator.

(vi) Reverse phase column 150 x 4.6 mm, Supleco Sil LC8, 5 µm diameter.

(vii) 0.45 µm cartridge Filter (Millipore, USA).

1.2.3. Method

(i) Mill feed samples through a 0.5 mm screen.

(ii) Extract with neutral detergent solution (sodium sulphite is not added because it solubilises phenolics).

(iii) Add 5 ml NaOH 2N and 0.1 ml nitrobenzene to 100 mg fresh weight of NDF residues in the tube.

(iv) Heat at 160°C for "3 h" in the oil bath - mix periodically.

(v) After cooling, wash the mixture out of the tube and dilute it to 50 ml with distilled water.

(vi) Extract 3 times *5* ml of diluted solution with 2 ml petroleum ether - acidify with concentrated H_2SO_4.

(vii) Dilute the final solution (2 ml) to 10 ml with distilled water - filter it through a 0.45 µm cartridge filter - inject into a HPLC.

(viii) Read peaks at "270 nm" - carry out reverse phase chromatography using a steel column and isocratic elution with water/methanol (76:24; v/v) acidulate - the flow rate is 1 ml/min and the column temperature is 32°C - injection volume is 20 µl each time.

(ix) Separate compounds are estimated by reference to a calibration curve of standard phenolic monomers.

1.2.4. Results

Nitrobenzene alkaline oxidation cleaves mainly the uncondensed linkages of lignin monomers and generally produces 8 compounds: p-hydroxyphenyl acid, p-hydroxybenzaldehyde, vanillic acid, vanillin, syringic acid, syringaldehyde, p-coumaric acid and ferulic acid. The monomers can be grouped into 3 units: hydoxyphenyl, guaiacyl and syringyl units.

The hydroxyphenyl units (p-hydroxyphenyl acid, p-hydroxybenzaldehyde) are usually in the middle lamella at an early stage of lignification and are highly condensed.

The guaiacyl units (vanillic acid and vanillin) are usually in the middle lamella at an early stage of lignification and are highly condensed and in the secondary wall at a later stage of lignification are lightly condensed.

The syringyl units (syringic acid, syringaldehyde) are present at the end of the lignification process within the secondary wall and are lightly condensed. With stage of maturity, syringyl units increase and guaiacyl units decrease and they can be considered both as an index of cell wall lignification and age of the plant.

1.2.5. Limits of this method

(i) Repeatability often higher than 5% - we suggest to triplicate analyses.

(ii) Problem of solubilization of free and loosely bound phenolics during neutral detergent extraction.

(iii) Variable yields of monomer products can be obtained with small variations of alkali oxidation conditions.

(iv) Temperature and mixing during boiling are critical steps in the analysis.

(v) Results give quantitative information only for uncondensed lignin but not all the core lignin is measured; the method is not useful in describing lignin profiles or for "fingerprinting" plant species.

2. Soluble phenol determination (Tempel, 1982; Mole and Waterman, 1987; Makkar, 1989; Scalbert, 1992)

Concerning soluble phenols several assays are mentioned in the bibliography. Selection of tests depends on:

(i) Plant tissue: tissue known to contain antinutritive factors or indigestible soluble molecules. Unusual results with classical measurements *in vivo* (% faecal nitrogen high against urinary nitrogen, diminution of ammonia concentration in the rumen, etc.).

(ii) Objects of the measurement: quantitative determination of total phenols, total tannins. Information about the structure of phenols, especially tannins. Information about biological activity of tannins and their effect on nutritional value. Sensitivity required.

(iii) Assays: facility, duration, reproducibility, number of extracts to analyse, equipment available, presence of interfering substances in the extract.

2.1. Quantitative determination and information about the structure of tannins (colorimetric determination)

All these methods have positive and negative points: simple, world admitted, standardized, but not specific, reacting more readily with monomers which are not tannins.

2.1.1. Quantitative determination

Folin-Denis (FD), Folin-Ciocalteu (FC) or Prussian blue (Burns, 1963; Price and Butler, 1977): determine total phenolics, simple method, reproducible but not specific (interferences with oxidizable plant constituents).

FC and FD are based on the reduction of a phosphotungstic-phosphomolybdic reagent in slightly alkaline medium. FC seems the most appropriate to determine absolute concentrations of complex phenolic mixtures. Linear response is obtained for absorbance >=0.5.

Variants exist to measure only polyphenols or total tannins (see 2.4.3).

2.1.2. Structure of tannins

(i) Vanillin-HCl method (see 2.4.5.1): vanillin, the most widely used aldehyde, gives a red chromophore with condensed tannins but reacts more readily with monomeric catechins which have no tanning properties. This method is rapid but only reproducible under strict assay conditions (temperature, light sensitivity, timing, etc. Several authors prefer vanillin-sulphuric acid method (Scalbert *et al.*, 1989). Butler *et al.* (1982) proposed a variant to determine polymer length.

(ii) n-Butanol-HCl method (see 2.4.5.2): a depolymerization treatment is applied to the estimation of proanthocyanidins. Hot mineral acid treatment transforms proanthocyanidins to anthocyanidins. It is a specific assay: it determines only condensed tannins (not monomeric forms) but also non-tannin monomeric flavanoids. Proanthocyanidins may be underestimated due to incomplete conversion into anthocyanidins.

(iii) Rhodanine assay (see 2.4.6): galloyl esters are hydrolyzed and the resulting gallic acid is estimated with rhodanine in alkaline medium. This test determines specifically gallotannins (one class of hydrolysable tannins) but with low molecular weight (non-tannins) too. Unhydrolyzed galloyl esters do not react.

(iv) Nitrous acid assay (Scalbert *et al.*, 1989): hexahydroxydiphenol groups react with nitrous acid and give a red or pink colour changing slowly to purple or blue. Ellagitannins and free ellagic acid

(monomer) are determined specifically. This method is low in sensitivity and not easy because it must be done in the absence of oxygen.

Depolymerization treatments exist for estimation of either ellagi- or gallotannins measuring the resulting monomer by chromatography. These methods are sensitive, no other phenols interfere with the results but they are time-consuming and require HPLC equipment.

Other techniques may precise quantities and composition of the phenolic profile of the plant extract. Generally these methods need extractive purification and/or special equipment: thin layer chromatography, high performance liquid chromatography, mass spectrophotometry, etc.

2.2. Biological activity of tannins (protein precipitation methods)

Methods are based on tannin-protein interactions or the so-called astringency property.

Insoluble proteins can be used:

(i) Hide powder (AFCIC norme 310-53): the principle is based on the percolation of a tannin solution in a column filled with hide powder: the amount of fixed tannins is measured by difference of DM before and after percolation. Used traditionally in the leather industry, this method is time-consuming, requires a large amount of tannins and overestimates tannin content.

Methods using soluble proteins are based on their precipitation in presence of tanning molecules. The result depends on the choice of the protein, protein concentration, sample's tannins, precipitation conditions, measured molecules (complexed polyphenols or proteins, free polyphenols or proteins). Furthermore, although phenolics of low molecular weight are too small to form the effective cross-links between proteins which result in precipitation of stable complexes, many of these have been shown to inhibit enzyme activity and to bond to proteins. Tannins-protein complexes can be partially soluble and not measured. The most used soluble proteins are:

(ii) Gelatine's AOAC method (1965): it is based on volumetric titration. Tannin concentration is obtained by substraction after titrating the total raw extract and then the extract after tannin removing: the tannins are removed from the raw extract by precipitation with an acidic-gelatine-salt solution. The indicator of phenols in suspension is potassium permanganate. This method requires minimal amount of equipment; there is no interference with methanol as extraction solvent; this method is standardized and relatively easy but time-consuming, not very sensitive, used only for high tannin-concentrated extract (>0.15 $g.l^{-1}$ tannins) and overestimates tannins. A variant of this method is proposed in 2.4.4.

(iii) Haemoglobin for haeme analysis (Bate-Smith, 1973): haemoglobin is measured (λ=578 nm) before and after precipitation by tannins. Results are expressed as relative astringency. This microtechnique is easy, very sensitive in the range of 0.3-0.8 mg tannic acid equivalents per ml of extract but needs fresh blood and evaporation of extract solvent; interferences from plant constituents are possible.

The most actually used protein is: *bovine serum albumin.*

(iv) BSA precipitation method (see 2.4.7): This test is easy but interferences with other plant constituents are reported in the bibliography. Several variants of this method exist (Hagerman and Butler, 1978; Makkar, 1989, etc.).

(v) Radial diffusion (see 2.4.8): this very easy method requires neither complex reagents nor instruments, is well adapted to a small amount of tannins (about 0.5 mg); non-tannin phenolics or the organic and aqueous solutions which are commonly used to extract tannins from plants interfere but, the same person has to measure rings of precipitation (subjective measurement).

For all these methods results may not be representative of the interactions of proteins and tannins in the plant sample of interest.

2.3. Choice of standards

Results can be expressed as OD units per g DM of the sample but they are often expressed in terms of the chosen standard. The most commonly used standards are relative standards especially tannic acid: this standard is appropriate as a chemical standard but quite inappropriate for bioassay (protein precipitation assays). Quebracho tannin is the only readily available condensed tannin. Absolute standards are difficult to obtain. Choose your own standards in regard with bibliographic results.

Choice of standard is proposed for each method explained in 2.4.

2.4. Choice of assays for soluble phenolic compounds

A choice of methods is proposed to have a good determination of the sample. The proposed protocols (sample conditioning, extraction, tests) are not always the most adapted but are the most reported in the bibliography.

Remarks: assays have to be done rapidly because the extract is unstable (it can be kept 1 or 2 days at 4°C). Quantities proposed could be not appropriate according to samples. Make adjustments, if necessary. Each result has to be reported to dry matter of the sample.

2.4.1. Sample conditioning

(i) Note growth stage.

(ii) Conditioning: every conditioning process affects more or less quantity and reactivity of tannins. Always precise every detail of storage. Measure tannins on fresh sample or like that offered to animals. If it is not possible, dry samples with soft and well-known conditions (aired room, in the shade, at room temperature) or freeze dry or put in air liquid (moisture causes decrease of quantity of tannins). Mill to 5 or 1 mm according to the sample.

(iii) Ether extraction if lipids >10% DM.

2.4.2. Phenolics extraction

(i) 70% acetone or 50% methanol. Acetone disrupts complexes formed between tannins and proteins better than methanol but interacts with some assays.

(ii) Sample/solvent (w/v) 10-20 g/l.

(iii) Extraction:

- at 4°C (in ice bath) with ultrasonication during 10 min
- or at room temperature during 1 h
- or at 4°C without agitation during 24 h

(iv) Centrifugation: 15 min 5000 g or filtration on crucible No. 2.

→ Supernatant (noted supernatant 1) (kept at 4°C and protected from light).

Remarks: for assays using supernatant 1 be careful with acetone because of evaporation. It is difficult to extract all phenols of the sample. The only solution is to exhaust the sample by successive extractions.

2.4.3. Assay of total phenols and total tannins: method based on Julkunen-Tiitto (1985)

(i) With 200 mg sample, after extraction, take 2 ml of supernatant 1 and add 100 mg insoluble PVP (polyvinylpyrrolidone).

(ii) Mix (vortex) and keep on ice 5 min - mix (vortex) again and keep 5-7 min on ice.

(iii) Centrifuge and collect supernatant 2 using Pasteur pipette.

Folin-Ciocalteu assay on supernatant 1 and 2:

Reagents: Folin reagent 1 N; sodium carbonate saturated solution: 20% Na_2CO_3; tannic acid standard solution freshly prepared (0.5 $mg.ml^{-1}$ of solvent).

(i) Take x µl supernatant 2 adjusted with distilled water to 1 ml.

(ii) Standard solution: take different volumes of this solution: 0 (blank) -20-40-60-80 µl (x2) and add distilled water to adjust to 1 ml.

(iii) Add 0.5 ml Folin reagent in each tube (blank, standard, samples) - mix (vortex) and wait 3 min.

(iv) Add 2.5 ml of 20% Na_2CO_3.

(v) Mix (vortex) and leave for 40 min at room temperature.

(vi) Read at 725 nm.

(vii) Express results as tannic acid equivalents:

- [total phenols] = [supernatant 1]
- [total tannins] = [supernatant 1] - [supernatant 2]

Remarks: when Na_2CO_3 is added in tubes, precipitation can occur: remove it by centrifugation or let precipitate during the 40 minutes of the assay. PEG (polyethylene glycol) or PVP can be used. Capacity of binding to polymer depend on its nature (PVP or PEG) and its molecular weight. Interferences with non-tannins are reported.

2.4.4. Assay of total tannins: with gelatine (Marigo, 1973)

Reagents: HCl and NaOH 0.1 N; gelatine solution (1% gelatine 10% NaCl in distilled water); tannic acid standard solution (0.5 mg ml^{-1} of distilled water or of solvent if there is no acetone).

(i) Remove solvent of supernatant 1 by evaporation if solvent contains acetone; if not, keep supernatant 1 for all assays.

(ii) Adjust to 100 ml = supernatant 1 bis.

(iii) Take 10 ml of this solution - adjust pH to 4.5 with HCl or NaOH 0.1 N.

(iv) Add 0.1 ml gelatine solution.

(v) Mix gently.

(vi) Add 0.1 ml gelatine solution one more time and until there is no more precipitation (if you add more than 0.5 ml gelatine solution, then dilute sample solution).

(vii) Centrifuge → supernatant 3.

(viii) Folin-Ciocalteu assay on supernatant 3 and on supernatant 1 bis (see above).

(ix) [total tannins] = [supernatant 1 bis] - [supernatant 3], expressed as tannic acid equivalents.

Remarks: the assay uses supernatant after removing acetone. It is not easy to determine the exact time when gelatine addition is not needed anymore. To compare total phenol measurement with total tannins, measure total phenols with supernatant 1 bis (without acetone).

2.4.5. Condensed tannins

2.4.5.1. Acidified vanillin method (Broadhurst and Jones, 1978)

Reagents: concentrated HCl; freshly prepared 4% vanillin (w/v) in methanol; methanol; catechin standard solution (catechin 0.5 mg ml^{-1} in methanol).

(i) Take 0.25 ml supernatant 1 bis or x µl adjusted to 0.25 ml with methanol (if x <75 µl, you can use supernatant 1).

(ii) Standard solution: 0 (blank)-15-30-...-75 µl of standard solution adjusted to 0.25 ml with methanol.

(iii) Add 1.5 ml vanillin solution - mix thoroughly.

(iv) Add 0.75 ml concentrated HCl - mix thoroughly.

(v) Cap the tubes and stand for exactly 15 min at room temperature.

(vi) Read OD of samples, standards and blank against water at 500 nm. Results are expressed as catechin equivalents.

(vii) To allow for interferences in the results due to the presence of anthocyanins, a parallel measurement should be made where methanol replaces vanillin solution. Read OD of samples at 500 nm. Absorbance due to anthocyanins is substracted from the first measurement.

Remarks: if the result is very low, it is not necessary to go further with the second assay (butanol-HCl method). Parallel measurement is necessary if the extract solution naturally absorbs at the wavelength of the assay. This could also be measured for butanol assay and for BSA precipitation.

2.4.5.2. (n)-Butanol-HCl assay

Reagents: acid butanol reagent: 5% HCl in butanol; iron reagent {ferric ammonium sulphate [$NH_4Fe(S0_4)_2$, $12H_2O$] 2% in HCl 2M}.

(i) Take 1 ml supernatant 1 or x µl adjusted to 1 ml with solvent.

(ii) Add 6 ml acid butanol reagent.

(iii) Add 0.2 ml iron reagent.

(iv) Mix (vortex) - cap the tube loosely and put in a boiling bath (95°C) for 40 min.

(v) Cool the tube and read absorbance at 550 nm against a blank (prepared from 1 ml of solvent instead of sample solution).

(vi) Results are expressed as absorbance at 550 nm per g dry matter or as standard equivalents (quebracho tannin or cyanidin).

2.4.6. Hydrolysable tannins: Gallic acid determination (Inoue and Hagerman, 1988)

Reagents: 2 N sulphuric acid; 0.667% rhodanine (w/v) in methanol (stable for 2 weeks at 4°C); 0.5 N KOH; gallic acid or tannic acid standard solution (see 2.4.4).

(i) 1 ml supernatant 1 bis or x µl adjusted to 1 ml of solvent.

(ii) Add 5 ml sulphuric acid in an ampoule.

(iii) The sample is frozen and the ampoule vacuum-seal and heat for 26 h at 100°C.

(iv) Cool the ampoule, open and make the content to 50 ml with water.

(v) Take 1 ml of this solution and add 1.5 ml rhodanine reagent - mix.

(vi) After exactly 5 min add 1 ml KOH.

(vii) After 2.5 min dilute the mixture to 25 ml with distilled water.

(viii) After 5-10 min read absorbance at 520 nm.

(ix) Results are expressed as gallic acid or tannic acid equivalents.

2.4.7. Bovine serum albumin precipitation (Hagerman and Butler, 1978)

Reagents: buffer (0.20 M acetate buffer with 0.17 M NaCl adjusted to pH 5.0 with NaOH); BSA solution (1 $mg.ml^{-1}$ in buffer); tannic acid standard solution (see 2.4.4); SDS-TEA solution (1% sodium dodecyl sulfate + 5% triethanolamine (v/v) in distilled water); ferric chloride reagent (0.01 M ferric chloride in 0.01 M HCl).

(i) 2 ml BSA solution + 1 ml supernatant 1 bis (or x µl adjusted to 1 ml with buffer).

(ii) 2 ml BSA solution + x µl standard solution adjusted to 1 ml.

(iii) Mix gently for 15 min at room temperature.

(iv) Centrifuge at 5000 g for 15 min.

(v) Wash pellet with buffer (2 ml) - mix for 15 min - centrifuge.

(vi) Dissolved precipitate with 4 ml of SDS-TEA solution.

(vii) Add 1 ml ferric chloride reagent.

(viii) After 15 min read absorbance at 510 nm and express results as absorbance at 510 nm/g of the material.

(ix) Compare phenol sample precipitation with tannic acid precipitation.

2.4.8. Radial diffusion (Hagerman, 1987)

Reagents: buffer A (50 mM acetic acid + 60 µM ascorbic acid adjusted to pH 5.0); agarose solution [agarose (type I) 1% (w/v) in buffer A by heating the suspension of agarose to boiling while stirring]; standard solution (catechin or tannic acid) in extraction solvent for example 62.5 mg ml^{-1} (0.50 mg/8 µl).

(i) Add protein (0.1% BSA w/v) to the agarose solution (cooled to 45°C) - mix (magnetic stirrer).

(ii) Dispense 9.5 ml aliquots into standard plastic petri dishes (8.5 cm diameter).

(iii) After cooling store plates at 4°C until utilization.

(iv) Punch wells (4.0 mm in diameter) spaced 1.5 cm apart on the plates (~ 5 wells/plate).

(v) Add tannin-containing solutions (supernatant 1 or standard solution) to the well with a micro-syringe (~ 8 µl/well x 4 times).

(vi) Cover and seal with parafilm the petri dishes.

(vii) Incubate at 30°C for 96 h.

(viii) Measure diameter of the rings: for each ring, 2 diameters at right angles must be measured.

(ix) Tannin concentration is calculated from the square of the average of the 2 diameters using the calibration curve.

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Estimation of organic matter digestibility of forages and feeds by pepsin-cellulase method

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1. Background

New laboratory methods for estimating organic matter digestibility have been developed to replace both the rather unreliable chemical methods and also biological methods, generally more precise but requiring fistulated animals. Among modern estimation methods, those using enzymes show certain advantages: they are rapid, reproducible and economical.

These methods are widely used for forages, and have also been applied to by-products, concentrate and mixed feeds produced by agro-food industry. For various types of forages, in temperate or tropical zones, prediction is higher than with chemical methods and comparable to that obtained *in vitro*. In addition cellulase methods can be used for mixtures and permanent pastures. They also offer the advantage of measuring improvement in the digestibility of hays and straws treated with ammonia, unlike the usual chemical methods.

For forages containing tannins, organic matter digestibility prediction is poor when cellulolytic enzymes are used. The enzymes are used at pH values different from those prevailing in the rumen, enabling possible release of tannins bound to proteins. Tannin-protein bonds favoured at the rumen pH (5 to 7) dissociate at pH values outside the range 4 to 7. In the pepsin-cellulase method, pepsin pre-treatment (pH approx. 3) could cleave protein-tannin complexes and lead to an overestimation of organic matter digestibility for these forages. (Aufrère and Guérin, 1996). Some kinds of tannins might have inhibiting effects on the enzyme activity especially that of cellulases.

2. Method (Aufrère and Michalet-Doreau, 1983)

Three stages are involved:

(i) Pretreatment by pepsin in hydrochloric acid (0.2% pepsin in 0.1 N HCl) in a water-bath at 40°C for 24 hours.

(ii) Starch hydrolysis in a water-bath at 80°C in the same solution for exactly 30 minutes.

(iii) Hydrolysis by cellulase 'Onozuka R10', in sodium acetate buffer (0.05M, pH 4.6) during 24 h at 40°C.

The residue is dried in an oven at 103°C, weighed and then mineralized in a furnace at 500°C for 4 hours, then reweighed. Digestibility by pepsin-cellulase is the percentage of dry matter or organic matter solubilized by the two treatments.

3. Reagents

(i) Hydrochloric acid 0.1 N.

(ii) Pepsin Merck 2000 FIP U/g Art 7190, store +4°C, solution 0.2% (w/v) n 0.1N HCl Dissolve 2 g/l pepsin in 0.1N HCl prewarmed at 40°C (instantaneously prepared solution).

(iii) Sodium acetate $3H_2O$ pH 4.6. Dissolve 6.8 g/l in distilled water.

(iv) Use concentrated acetic acid to adjust pH of sodium acetate buffer.

(v) Cellulase "Onozuka R10" extracted from *Trichoderma viride* (store +4°C) Medicine Department Yakult Honskha Ltd No. 1-19, 1-Chome, Higashi-Shinbashi, Minato-Ku, Tokyo 105, Japan, cellulase solution (1 g/l). Dissolve 1 g of cellulase in one litre of sodium acetate buffer prewarmed at 40°C (instantaneously prepared solution). Weigh cellulase with a mask to avoid allergy.

(vi) Glass powder. Glass powder G1 (ref 58509) VERAL sarl-46, rue Rouget de Lisle, BP No. 16, 92702 Colombes Cedex (Tel: 42 42 88 88).

4. Apparatus

(i) Precise balance 1/10 mg.

(ii) Water-bath without agitation set to 40°C ± 1°.

(iii) Water-bath without agitation set to 80°C ± 1°.

(iv) Thermostatted muffle furnace.

(v) Oven set to 103°C ± 1°.

(vi) Desiccator with dehydrating agent.

(vii) Crucibles with filtering plate (porosity 2) (see Fig. 1).

(viii) Stoppers No. 10 or 38D (∅ at the bottom 30 mm, ∅ at the top 36 mm) (Prolabo).

(ix) Stoppers No. 31D (Prolabo).

(x) Vacuum pump.

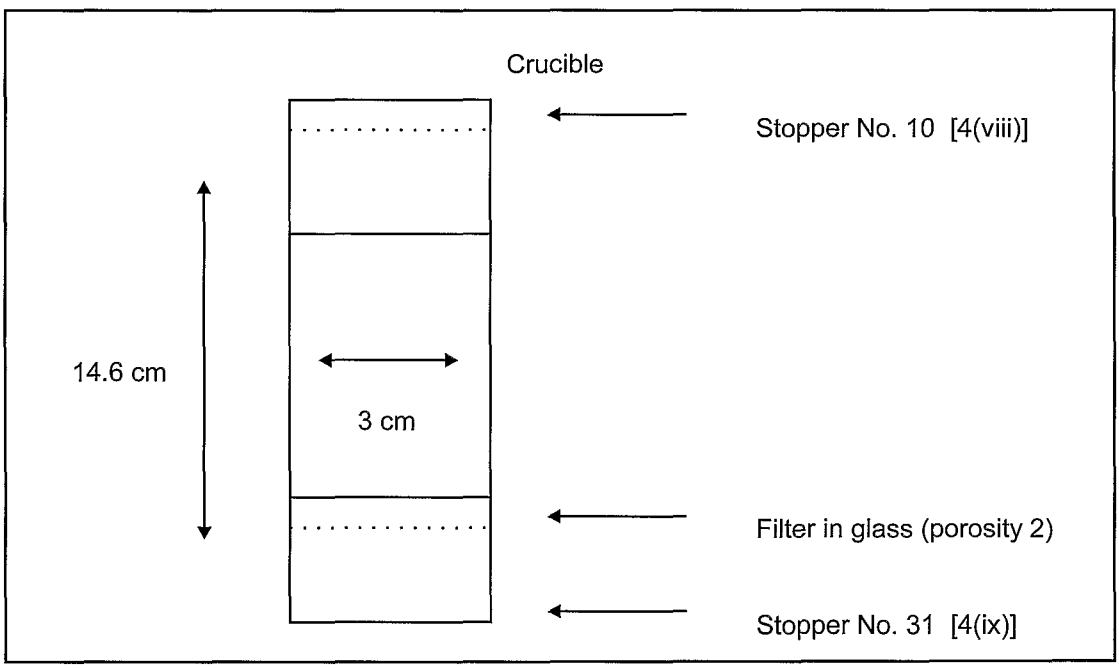

Fig. 1. Crucible with filtering plate.

5. Procedure

5.1. General procedure

(i) The samples are ground at 0.8 or 1 mm.

(ii) The analyses are performed in triplicate.

(iii) It is advised to use no more than 36 or 40 crucibles (3 holders) in a series.

(iv) Drying crucibles. First, crucibles are incinerated in a furnace at 500°C. After drop of temperature in a desiccator, weigh the crucible. Weight of empty crucible: P0.

(v) Weigh exactly 500 mg of sample in each crucible.

(vi) Simultaneously determine on each sample in duplicate the content of dry matter of sample (48 h at 103°C); and the content of ashes according to the norm NF 18 101.

(vii) In each series introduce a standard.

5.2. First step

In each crucible containing sample and closed at the bottom with a stopper [4(ix)], introduce 50 ml of pepsin HCl 0.1 N at 40°C [3(ii)] and closed with a stopper No. 10 [3(viii)]. Stir. Incubate the crucibles in a water-bath at 40°C [4(ii)]. Stir three times a day.

5.3. Second step

(i) After 24 h, incubate the crucibles exactly 30 minutes in a water-bath at 80°C [4(iii)] (remove before all the stoppers No. 10 [4(viii)].

(ii) Incubate holder after holder with interval in the time to facilitate the filtration which happens exactly after 30 min hydrolysis.

(iii) Filter rapidly all the crucibles to avoid an additional hydrolysis, then rinsing every crucible with about 250 ml of distilled water (about three times 80 ml) and finish with rinsing the bottom of crucible (under the filtering plate).

5.4. Third step

(i) Close the crucibles at the bottom with stoppers [4(ix)].

(ii) Incubate each crucible with 50 ml of prewarmed (40°C) sodium acetate buffer with cellulase [3(v)].

(iii) Close crucible and stir three times a day.

(iv) Incubate the crucibles in a water-bath at 40°C.

5.5. Fourth step

(i) After 24 h, filter each crucible. Rinse the residue with about 250 ml of distilled water (about 3 times 80 ml) and finish with rinsing the bottom of crucible (under the filtering plate). Dry the residue 48 h at 103°C then weigh: Weight of crucible + Residue (in dry matter) = P1.

(ii) Cook the crucibles during 5 h in the furnace at 500°C (not exceeding this temperature because crucibles may melt).

(iii) Weigh crucible + ashes = P2.

To avoid problem of filtration (with some samples) add 1 g of glass powder [3(vi)] and do not forget to take it into account in the calculation.

6. Calculation

P0 = weight of empty crucible
P1 = weight of empty crucible + residue (in dry matter)
P2 = weight of empty crucible + ashes

E(DM) = weight of sample (in dry matter)
E(OM) = Sample (in organic matter)
Dcell MS = cellulase digestibility of dry matter
Dcell MO = cellulase digestibility in organic matter

$$\text{Dcell MS} = \frac{\text{E(DM)} - (\text{P1} - \text{P0})}{\text{E(DM)}} \times 100$$

$$\text{Dcell MO} = \frac{\text{E(OM)} - (\text{P1} - \text{P2})}{\text{E(OM)}} \times 100$$

It is acceptable to find a range of 2 points with the standard we use in the series. If the value of the standard is out of this range, you must begin the series again.

7. Limitations

For tree foliage species with or without tannins, pepsin-cellulase digestibility is closer to *in vitro* digestibility than chemical criteria in general, though its accuracy is very poor. Both enzymatic and chemical methods lead to satisfactory accuracy only if the different species are taken separately. Besides their ease of use, and their favourable repeatability and reproducibility, these enzymatic methods allow a satisfactory classification of forages and accurate prediction of digestibility except for forages containing tannins for which adaptations are necessary.

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Estimation of *in situ* degradability of feed proteins in the rumen by a laboratory method using a protease

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1. Background

The quantity of feed proteins reaching the small intestine of ruminants depends on the total protein content of the feed and its degradability in the rumen. *In vivo* measurements (duodenal cannulation) are usually the standards, but are expensive and time consuming. The method most closely related to the environment in which the protein degradation takes place is the nylon bag method (*in situ* method) as described by Ørskov and McDonald (1979).

Other simple and cheaper laboratory methods have been developed. Among them, the use of enzyme preparation appears promising. These methods provide a good prediction of the degradability of feed proteins of by-products, concentrate and mixed feeds produced by agrofood industry. They are cheap compared with the reference methods (*in sacco* measurements) and offer the following advantages: there is neither microbial contamination of bag residues nor loss of particles through the bag mesh.

The application of these methods to forages containing tannins raises certain problems. In our method, the commercial protease of bacterial origin is used in a borate-phosphate buffer at pH 8. At this pH the enzyme activity is optimum but the tannin-protein complexes are unstable, which may cause an overestimation of the true enzyme degradability.

2. Method (Aufrère and Cartailler, 1988)

The sample is hydrolyzed by a protease in a borate phosphate buffer at pH 8 at 40°C for 1 hour. The determination of nitrogen content is performed on the supernatant and calculated in comparison with the quantity of total nitrogen of sample.

3. Reagents

(i) Sodium dihydrogen phosphate dihydrate, $NaH_2PO_4\ 2H_2O$.

(ii) Sodium tetraborate decahydrate, $Na_2B_4O_7\ 10H_2O$.

(iii) Protease Sigma P5417 extracted from *Streptomyces griseus* (type XIV), store +4°C. Check the activity of enzyme when you use a new batch of enzyme (see method described in appendix).

(iv) NaOH 2M/l.

(v) Borate-phosphate buffer pH 8. Dissolve 12.2 g of sodium dihydrogen phosphate [3(i)] and 8.91 g of sodium tetraborate [3(ii)] in one litre of distilled water. Adjust at pH 8 with NaOH [3(iv)].

(vi) Enzymatic solution of protease at 900 UI/100 ml (see measure of enzymatic activity in the appendix). Dissolve by magnetic agitation during 10 mn, an adapted weight of protease [3(iii)] in about 90 ml of buffer [3(v)] to obtain a solution at 900 UI/100 ml. Adjusted to 100 ml. Filter on filter paper [4(v)] (instantaneously prepared solution).

(vii) Enzymatic preparation (4.5 UI/sample). This solution is prepared by dilution 0.5 ml of enzymatic solution [3(vi)] in 50 ml of buffer [3(v)].

4. Apparatus

(i) Water-bath without agitation set to 40° ± 1°C.

(ii) Centrifuge tubes of 80 ml (height 11.5 cm, exterior diameter 4 cm).

(iii) Stoppers for tubes [4(ii)].

(iv) Centrifuge for centrifuge tubes [4(ii)] (2100 g).

(v) Filters Whatman No. 41.

(vi) Filters Durieux ref. 2B or equivalent.

(vii) Oven set to 103°C ± 1°C.

(viii) Desiccator with dehydrating agent.

5. Procedure

(i) The samples are milled through a 0.8 or 1 mm screen.

(ii) The analyses are performed twice.

(iii) Weigh exactly 500 mg of sample in each tube [4(ii)].

(iv) Simultaneously determine the content of dry matter in each sample in duplicate (48 h at 103°C). In each series introduce a standard and a blank (a tube without sample is added to measure the nitrogen content of enzymatic preparation).

(v) Add in each tube 50 ml of prewarmed (40°C) enzymatic preparation [3(vii)]. Close the tubes and incubate them in the water-bath [4(i)]. The tubes are stirred by gently manual agitation at 0 mn and 55 mn.

(vi) At 55 mn, remove the tubes from water-bath and immediately centrifuge them at 2100 g, for 5 mn. Filter on filter [4(vi)]. Take 20 or 25 ml of the total supernatant to determine the nitrogen content (for calculation, not forget to take into account the volume).

6. Calculation

Enzymatic degradability at 1 h is the ratio between content of degraded nitrogen and content of total nitrogen.

Take into account the mean between 2 measurements if conditions of repeatability get satisfaction and express the results in %.

DE1 = S nitrogen x 100/T nitrogen

S nitrogen: % solubilized nitrogen = (14 x Tx (V-V0)/m)10*
T nitrogen: % total nitrogen
T: normality of hydrochloric acid
V: necessary volume of hydrochloric acid to the neutralization of studied sample
V0: necessary volume of hydrochloric acid to the neutralization of blank
m: weight of studied sample expressed in, dry matter
*Take into account the volume used for measurement

It is acceptable to find a range of 2 points with the standard we use. If the value of the standard is out of this range, you must begin the series again.

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Appendix

Measurement of enzymatic activity

1. Principle

Casein $\xrightarrow[\text{protease}]{}$ Amino acids

2. References

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Folin, O. and Ciocalteu, V. (1929). *J. Biol. Chem.*, 73: 627

3. Conditions

Temperature 37°C, pH = 7.5, Absorbance 660 nm.

4. Method colorimetric

(i) 50 mM Potassium Phosphate buffer, pH 7.5 at 37°C (store +4°).

- PM = 228.2. Weigh 1.141 g potassium phosphate (K_2HPO_4 $3H_2O$) (Sigma No. P-5504) in 90 ml distilled water. Adjust at pH 7.5 at 37°C with HCl 6N. Adjust to 100 ml

(ii) Casein solution 0.65%.

- Do not store this solution at +4°C because conserving is not good, to test enzymatic activity, 50 ml are enough.
- Weigh 0.325 g (50x0.65) of casein (Sigma No. C-0376 or C7078) in 50 ml potassium phosphate buffer. Heat gently (in a water-bath). Do not exceed the temperature to avoid hydrolysis of the solution.
- Adjust again at pH 7.5 at 37°C (if necessary) with NaOH 1N or HCl 1N. The solution is hazy, filter on Whatman paper No. 41.

(iii) Trichloroacetic acid reagent, 110 mM.

- 9 ml TCA 6. 1N (Sigma No. 490-10) in 500 ml distilled water, or
- weigh 1.8 g TCA in 100 ml distilled water to obtain a solution at 0.1102N.

(iv) Folin and Ciocalteu's Phenol reagent.

- 10 ml of reagent (Sigma No. F-9252) in 40 ml distilled water (instantaneously prepared solution).

(v) Sodium carbonate 500 mM (store +4°).

- PM = 106. Weigh 10.6 g sodium carbonate (Na_2CO_3) (Sigma, No. 5-2127) in 200 ml distilled water.

(vi) Sodium acetate 10 mM + calcium acetate 5 mM (store +4°C).

- Sodium acetate: PM = 136.08
- Calcium acetate: PM = 158.2
- Weigh 1.361 g of sodium acetate (NaC_2CO_3-$3H_2O$) Sigma (No. S-8625) and 0.791 g calcium acetate ($Ca(C_2H_3O_2)_2$) (Sigma, No. C-1000) in 900 ml of distilled water. Adjust the pH to 7.5 at 37° with 0.1 M acetic acid or 0.1 N NaOH. Adjust to 1000 ml

(vii) 1.1 mM L-tyrosine.

- PM =181.2. Weigh 199.3 mg (Sigma No. T3754) in 1000 ml distilled water. Heat gently (in a water-bath). Do not overheat 37°C and cool to room temperature

(viii) Protease enzyme solution.

- Immediately before use, weigh about 40 mg in 50 ml sodium and calcium acetate and prepare dilution in the same buffer (2 ml in 50 ml) to obtain between 0.1 and 0.2U/ml

5. Procedure

	Sample	Blank
Casein	5 ml	5 ml

Incubate the tubes at 37°C for about 5 mm. Then add:

Protease	1 ml

Mix well and incubate at 37°C for exactly 10 mm. Then add:

TCA 110 mM	5 ml
Protease	1 ml

Mix and incubate at 37°C for about 30 mn.
Filter with Whatman filter (50) and use the filtrate in colour development.

6. Colour development

	Std1	Std2	Std3	Std4	Blank	Sample or blank sample
Tyrosine 1.1 mM	0.05	0.10	0.20	0.40	0	2
Distilled water	1.95	1.9	1.8	1.6	2	0
Na_2CO_3	5	5	5	5	5	5
Folin reagent	1	1	1	1	1	1

All the volumes are expressed in ml.
Mix well and incubate at 37°C for 30 mn. Remove the tubes and allow them to cool to room temperature. Read the absorbance at 660 nm.
If the solutions are hazy, filter through a 0.45 µm.

7. Calculation

Optical density: OD
OD standard = ODstd - OD blank
OD sample = OD sample - OD blank sample

$$\text{Units / mg} = \frac{(\mu\text{mole Tyrosine equivalents release}) \times 11}{(\text{mg enzyme / volume(buffer)}) \times 10 \text{ mn} \times 2}$$

Unit definition
One unit will hydrolyze casein to produce colour equivalent to 1.0 µmole (181 µg) of tyrosine per minute at pH 7.5 at 37°C (colour by Folin and Ciocalteu's reagent).

8. Limitation

For tree foliage rich in tannins, the enzymatic method enables the accurate ranking of species and organs and can be used for screening.

The *in vitro* gas production technique

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1. Background

In most laboratory techniques used for feed evaluation, the disappearance or solubilization of substrate is measured. On the other hand, the gas production technique which was originally developed by Menke and Steingass (1988) measures the evolution of gases (methane and CO_2) which are produced as end products of fermentation. Production of CO_2 is partly from the fermentation and partly as a result of formation of volatile fatty acids which expel CO_2 from the carbonate buffer solution.

The gas technique provides a great advantage in that the fermentation takes place in a glass syringe which allows several measurements to be made on the same sample by measuring the gas volume at different intervals of time. This means that not only the possible extent of fermentation but also the rate of fermentation can be measured. In this respect the technique has similarity to the nylon bag and the same exponential equation can be used. Thus the gas technique complements the nylon bag technique by measuring end product formation and not substrate disappearance. Results from studies using this approach to predict animal performance (digestibility and intake) showed that the gas technique was slightly inferior to the nylon bag but a much better predictor than other *in vitro* techniques or chemical components of feeds (Blummel and Ørskov, 1993; Khazaal *et al.,* 1993; Dentinho *et al*., 1994).

Recently, the fact that the gas technique differs from other *in vitro* techniques by measuring evolution of gas as a result of fermentation, is advantageous in that it can be adapted as an excellent biological assay to estimate the level of phenolics-related antinutritive factors in feed (Khazaal and Ørskov, 1994; Khazaal *et al.*, 1994). This is achieved by adding phenolic binding agents such as Polyvinylpyrrolidone or Polyethylene glycol to the substrate. As a result the phenolics-related antinutritive compounds bind to the phenolic binding agent and their negative effects on fermentation are lessened.

2. Apparatus

The apparatus used in the gas production technique may vary slightly from one laboratory to another. At the International Feed Resources Unit (IFRU) the apparatus used is simple (see Fig. 1). It consists of glass syringes of 100 ml capacity which are incubated in a water bath where the temperature is accurately controlled with a water stirring heater. Therefore, the following procedure is based on the apparatus used in our laboratory.

2.1. Syringes

Good quality syringes are essential (syringes of Haberle Labortechnik, 7901 Lonsee-Ettlenschien, Oberer Seesteig 7, Germany are recommended). The syringes and their pistons should be numbered with a permanent (waterproof) dye starting, for example, with number 1. A few extra syringes are left without numbering as replacement for broken syringes.

2.2. Buffer solution

Stocks of the main elements solution (pH 6.8), the buffer solution (pH 8.1), the resazurin solution and the trace element solution can be prepared and stored in dark bottles. The reduction solution

must be freshly prepared. Preparations of all solutions are as described in page 9 by Menke and Steingass (1988). The pH of the buffer mixture solution (i.e., main elements + buffer solution + resazurin solution + trace element + reduction solution should be about 7.1 ± 0.15.

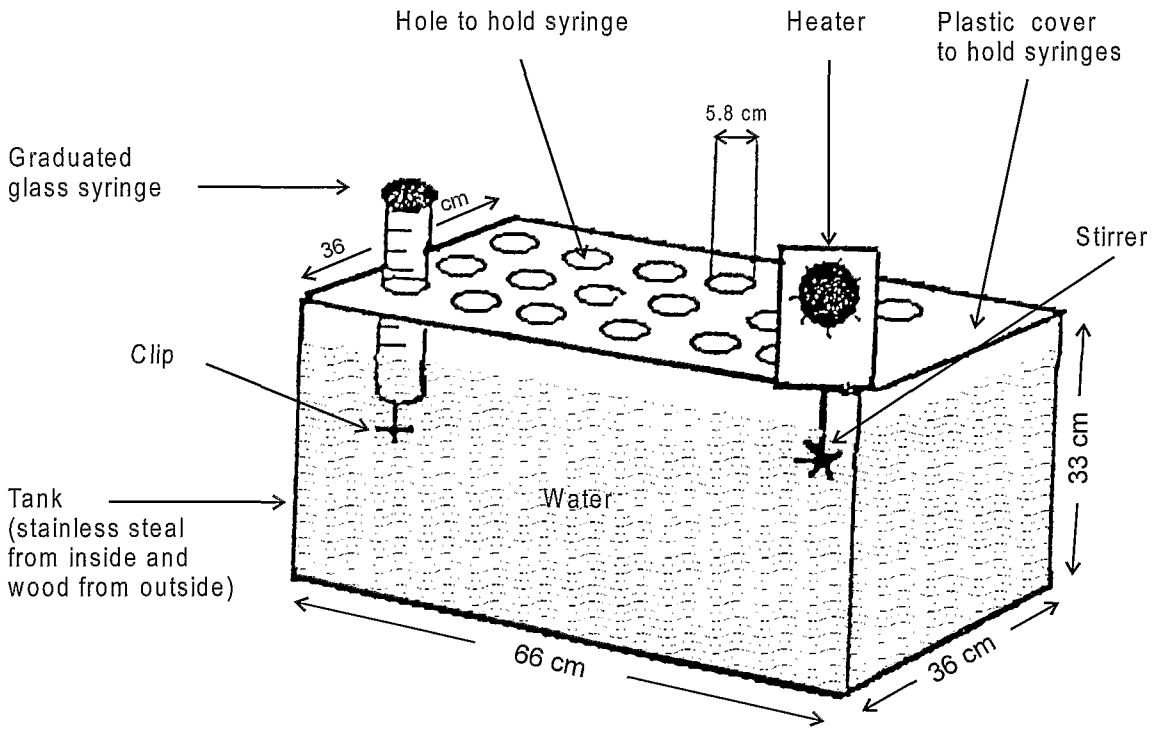

Fig. 1. Diagram of the gas production apparatus used at the International Feed Resources Unit.

2.3. Preparation of samples

The samples are milled through a 1.0 mm screen and their DM content determined.

Before weighing the samples, the position of the syringes in each run should be planned. Ideally there should be 3 replicates of a blank and a standard hay[†] in each run. The triplicates of the blank or the standard hay should be dispersed between the syringes. Thus one of each is incubated as your first (No. 1) and second (No. 2) syringes, the second blank and standard will be in the middle, while the third replicates of the blank and standard hay should be the last 2 syringes (see Table 1). Samples are normally duplicated/run and a run is usually repeated 3-4 times.

Zero the weight of the glass boat and then weigh 215 ± 5 mg of 1.0 mm-milled sample (air dry) (i.e., gives approximately 200 mg DM if the DM content of the samples is about 90%) in the glass boat and record the weight. Use an aluminium or metal rod to hold the glass boat containing the sample. Empty the content of the glass boat into the bottom of the glass syringe. Try not to get any particles or dust from the sample onto the high inner side of the syringe since this could affect the movement of the piston.

[†]According to Menke and Steingass (1988), a standard hay is used to correct for variations between runs and to give an indication if the run was successful or not. For this purpose, a local good quality hay may be produced and used. However, from our experience, variations between runs are small and therefore we do not use the standard hay to correct for variations between runs. A variation of ±5% in the volume of gas produced from the standard hay after 24 h incubation is acceptable. If variation is larger, the run should be repeated.

Table 1. Preparation of samples

Syringe number	Sample	Fresh weight	Level of piston at Zero time†	Gas production after 3 h, 6 h ... 96 h
1	Blank	Empty	About 30	
2	Std. hay	215 ± 5 mg	.	
3	Sample A	.	.	
4	Sample B	.	.	
5	Sample C	.	.	
6	.	.	.	
.	.	.	.	
.	.	.	.	
.	Sample n	.	.	
.	Blank	Empty	.	
.	Std. hay	215 ± 5 mg	.	
.	Sample n	.	.	
	.	.	.	
.	.	.	.	
.	.	.	.	
.	Sample C	.	.	
.	Sample B	.	.	
.	Sample A	.	.	
.	Blank	Empty	.	
n	Std. hay	215 ± 5 mg	.	

†i.e., after injecting rumen liquor:buffer mixture

Lubricate the pistons with a small amount of vaseline (pure petroleum jelly) to ease the sliding of pistons and prevent gas escape. Push the piston inside the glass syringe gently after opening the clip. Make sure the sample in the syringe is not blown up and that it does not come into contact with the piston.

At IFRU, weighing of samples into the syringes and lubrication with vaseline are completed the night before the start of incubation (run). The syringes are then prewarmed in an incubator 40°C overnight before the rumen liquor:buffer solution is injected into the syringe.

If a water bath is used, the heater should be turned on some time before the start of incubation (e.g., the night before).

2.4. Starting the incubation

It is important first to calculate roughly how much of the buffer mixture solution and rumen liquor is needed. This will depend on the number of syringes to be incubated. For instance if a total of 35 syringes are to be injected, i.e., at least 30 x 35 = 1050 ml of rumen liquor:buffer solution will be needed. In order to be on the safe side it is better to prepare at least 1200 ml of the mixture and for that 800 ml buffer and 400 ml filtered rumen liquor is needed. Therefore, prepare the 800 ml of the buffer mixture solution first in a Wolf flask. Heat the content of the flask to 39°C and then transfer it to the small water bath. Pump a submerged stream of CO_2 into the liquid until it becomes colour-less or very slightly pinkish. Then, lift the stream of CO_2 to a level above the surface of the liquid. It is important that the stream of CO_2 is lifted to prevent oversaturation of the buffer mixture with CO_2. If this is allowed to happen more CO_2 gas will be released as a result of buffering the volatile fatty acids during fermentation and as a result the variability between runs will increase. The reduction solution is added minutes before the addition of the rumen liquor.

2.5. Rumen liquor

The donor animals could be cattle, sheep or goats but should be receiving a good quality roughage-based diet (at IFRU, 3 rumen cannulated sheep are used and they receive 1200 g of hay:grass cubes (2:1) in two equal feeds per day). Before the morning feeds, equal amounts of rumen liquor from each of the 3 sheep is pumped into plastic bottles and quickly stored in warmed thermos flask and taken to laboratory. Then, the rumen liquor is stirred and filtered through 2 layers of Muslin. The filtered rumen liquor is bubbled with a stream of CO_2 for 1-2 minutes. This is followed by adding the required amount of filtered rumen liquor (pH 6.3 ± 0.15) while stirring the buffer solution in the flask. Remember that the proportion of rumen liquor to buffer is 1:2 (the pH of the rumen liquor:buffer mixture should be about 6.90 ± 0.1).

Remark: With poor quality roughages (i.e., crude protein less than about 6%) Casein (1.0 g) should be added to the buffer mixture as a source of protein for microbial growth.

2.6. Inoculation

Record the Zero time (i.e., the time when injection of the rumen liquor:buffer mixture into the syringes is started) of incubation.

Inject 30 ±1.0 ml of rumen liquor:buffer mixture into each syringe, followed by drawing most of the air from the syringe. Shake the syringe gently to make sure that all the substrate is mixed with liquid and then take out all remaining air or air bubbles from the syringe. Record the level of the piston (i.e., should be around 30.0 ml) and incubate the syringe in a water bath (39 ± 0.1°C).

Record the time when you finish filling the syringes with the rumen liquor:buffer mixture. The period of time needed to complete the filling of all syringes with the rumen liquor:buffer mixture should be as short as possible. At IFRU it takes about 15-20 minutes to complete 54 syringes.

Shake the syringes gently 30 min after the start of incubation and then every hour during the first 8-10 h of incubation. This is important when low quality roughages, which tend to float, are studied. When gas production is to be recorded, shake the syringes after taking the reading.

Normally, the time required to inject the rumen liquor:buffer mixture into the syringes is longer than that required to read the volume of gas production during incubation. This difference, particularly if highly fermentable feeds are studied, can lead to an over estimation of fermentation of feeds that received the inoculum first compared with those which receive the inoculum last. Therefore, when gas production is recorded at any incubation period, it is best that the time during which the readings are made is similar to that taken when the syringes were inoculated with rumen liquor:buffer. For example, if it took about 30 seconds to fill up each syringe with mixture, then you should allow about 30 seconds for reading gas volume for each syringe.

2.7. Duration of incubation

The duration of incubation should be long enough to allow for the description of the curve of gas production fully (i.e., until the curve reaches a plateau or until the difference in gas production between the last two incubation times is small). At IFRU, 96 h incubation is considered to be sufficient in most cases. The accumulating volume of gas is recorded after incubation periods of 3, 6, 12, 24, 48, 72 and 96 h. If gas production exceeds 60 ml for a sample, take the syringe out of the water bath and empty the silicon tube from water. Turn the syringe upwards, open the clip and push the piston to release the gas. The piston could be pushed until it is close or back to the 35 ml position. Record the new level of piston and resume the incubation.

2.8. Calculation

Subtract the volume of gas produced from the blanks (i.e., average of 3 replicates) from the volume of gas produced in each sample. This will be the observed volume of gas per X amount of

fresh sample. Then, knowing the DM content of each sample, the volume of gas per 200 mg DM could be calculated.

Data of gas production are then fitted to the exponential equation $p = a + b\ (1\text{-}e^{-ct})$ of Ørskov and McDonald (1979); p represents gas production at time t, (a + b) the potential gas production, c the rate of gas production and a, b and c are constants in the exponential equation. These characteristics of the fermentation of feeds have been successfully used in the prediction of animal performance (Blummel and Ørskov 1993; Khazaal *et al.,* 1993; Dentinho *et al.,* 1994).

Remark: it is acceptable to find that replicates of the blank or the standard hay that were first injected with rumen liquor:buffer mixture produce slightly less gas (1-1.5 ml) compared with the ones that were last injected. It is not very clear why this happens. One possible explanation is that during injection of the rumen liquor:buffer mixture, more particles accumulate at the bottom of the flask as the content of the Wolf flask becomes smaller. Another more likely explanation is that the rumen liquor:buffer mixture becomes increasingly saturated with CO_2 towards the end of inoculating the syringes compared to the start. This is why it is important to place syringes of blank and standard at different positions in each run.

3. Limitations of the technique

(i) In order to use the technique reliably it is essential to be sure of a constant supply of electricity in order to maintain a constant temperature during incubation.

(ii) Like other *in vitro* techniques, it is a closed system in which end products accumulate and can inhibit fermentation or create an environment very dissimilar to the rumen.

(iii) The technique will underestimate the nutritive value of feeds that are high in protein. This is due to the fact that protein fermentation contributes little to the total volume of gas production.

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Final considerations

The development of husbandry in the Mediterranean region under economically-efficient and ecological conditions requires optimizing the use of local forages.

Nevertheless, given the state of the art, available data on nutritive value of plants and shrubs from hot arid climates are not sufficient.

This generates a tendency to use imported products from humid temperate areas for animal feeding, thus greatly depressing the competitiveness of Mediterranean husbandry. This runs against *rational management of the plant biomass produced in this area. The determination of assessment* methods for nutritive value of forages has led to a proposal of recommended methods, thus enabling us to gain some knowledge on this matter.

Nevertheless it is probable that future research will improve assessment techniques, and this is to be hoped. But for the time being, this work is expected, through the homogenization of investigation methods, to provide the opportunity of sharing the results obtained in the different laboratories in order to better valorize local forage productions.

Considérations finales

Le développement de l'élevage dans la zone méditerranéenne dans des conditions économiques et écologiques nécessite l'optimisation de l'utilisation des fourrages locaux.

Toutefois dans l'état actuel de nos connaissances nous n'avons pas suffisamment d'information sur la valeur nutritive des plantes et arbustes des régions à climat chaud et aride.

Cela incite à utiliser pour l'alimentation du cheptel des produits importés des zones tempérées et humides, ce qui diminue fortement la compétitivité de l'élevage méditerranéen et va à l'encontre d'une gestion raisonnée de la biomasse végétale produite dans cette zone. La mise au point de méthodes d'évaluation de la valeur nutritive des fourrages ayant abouti à la proposition de méthodes recommandées devrait permettre d'augmenter nos connaissances dans ce domaine.

Certes il est probable que les recherches futures sont susceptibles d'améliorer les techniques d'évaluation et cela est bien souhaitable. Mais dès maintenant il est possible d'espérer que ce travail, grâce à l'uniformisation des méthodes d'investigation, permettra de mettre en commun les résultats obtenus dans les différents laboratoires afin de mieux valoriser les productions fourragères locales.