# Relationship between chemical composition and in vitro digestibility of some Spanish browse plants species

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# Relationship between chemical composition and *in vitro* digestibility of some Spanish browse plant species

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**SUMMARY –** Chemical composition and *in vitro* digestibility (IVD) were determined in 22 samples of leaves and flowers of six browse plant species (*Erica australis, Cistus laurifolius, Quercus pyrenaica*, *Cytisus scoparius*, *Genista florida* and *Rosa canina*) to examine the correlation between some chemical fractions and the digestibility of these fodder resources. Samples were analysed for extractable phenols, tannins, and condensed tannins. The activity of phenolic compounds was assessed as the increase in the *in vitro* gas production after incubation in rumen fluid in response to the addition of polyethylene glycol (PEG). IVD was positively ($P<0.01$) correlated with the crude protein content, showing a negative relationship with the ADF and lignin contents. Digestibility and tannin concentrations were negatively correlated showing inverse linear relationships with either the tannin concentrations or their biological activity assessed using PEG. Thus, lignin and tannin seem to be the main constraints of digestibility of browse plant species.

**Keywords:** Browse plants, tannins, digestibility, nutritive value.

***RESUME –*** "*Corrélation entre la composition chimique et la digestibilité* in vitro *de quelques espèces arbustives de l'Espagne*". *La composition chimique et la digestibilité* in vitro *(IVD) ont été déterminées dans 22 échantillons de feuilles et de fleurs de six espèces arbustives (*Erica australis, Cistus laurifolius, Quercus pyrenaica, Cytisus scoparius, Genista florida *et* Rosa canina*) pour examiner la corrélation entre quelques fractions chimiques et la digestibilité de ces ressources fourragères. Les phénols extractibles, les tannins et les tannins condensés ont été analysés sur les échantillons. L'activité des composés phénoliques a été évaluée à partir de l'augmentation de la production de gaz après l'incubation* in vitro *en jus de rumen en réponse à l'addition de polyéthylène glycol (PEG). L'IVD a été positivement corrélée ($P<0,01$) avec la teneur en protéine brute, montrant une corrélation négative avec le contenu d'ADF et de lignine. La digestibilité et les concentrations de tannins ont été négativement corrélées, montrant des rapports linéaires inverses avec les concentrations de tannins ou leur activité biologique évaluée en utilisant le PEG. Ainsi, la lignine et les tannins semblent être les contraintes principales de la digestibilité des espèces arbustives.*

***Mots-clés :*** *Espèces ligneuses et arbustives, tannins, digestibilité, valeur nutritive.*

## Introduction

Browse species are widely distributed throughout the Mediterranean basin and represent a significant resource for the large population of ruminants in this area. Foliage from these species may represent a high proportion of the food ingested by sheep, cattle and goats (Van Soest, 1994). Many of the browse species contain variable amounts of structurally diverse secondary compounds such as phenolics, tannins and other compounds with anti-nutritional properties, limiting the feeding value of shrubs and tree fodders for ruminants (Kumar and D'Mello, 1995). Nutritional and metabolic effects of tannins range from beneficial to toxic to the animal (Min *et al*., 2003). Little is known about the relationship between the biological reactivity and the chemical structure and composition of tannins, partly due to the diverse and complex nature of these molecules. For a quick assessment and initial screening of tannins in browse species, relatively simple bioassays have been proposed. One of these assays is based on *in vitro* rumen fermentation incubations coupled with the use of a tannin-complexing agent such as polyethylene glycol (PEG). However, there are few studies investigating the relationship existing between the analytical methods and the biological activity of tannins. The main objective of the present study was to examine the relationship between the chemical composition, especially the tannin concentration, and the *in vitro* digestibility of some Spanish browse plant species.

## Material and methods

### Source of shrubby samples

Twenty two samples consisting of leaves and flowers from six browse species were collected at different times from spring to autumn in 1998, so plants were at different vegetative maturity stages. The browse species were: *Erica australis*, *Quercus pyrenaica*, *Cistus laurifolius*, *Cytisus scoparius*, *Genista florida* and *Rosa canina*. The selection of the species was based on the available information about their palatability and preference by small ruminants and on their relative abundance of the different shrub and tree fodders in the area of study, the uplands of the province of León (Norwest of Spain). Branches and twigs of several specimens of each species were clipped with scissors harvesting a mixture of leaves, flowers and fine green stems. In the laboratory, leaves and flowers (when available) were separated by hand from the original samples, immediately freeze-dried and ground in a hammer mill using a 1-mm sieve for subsequent analysis. Crude protein (CP), neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin were determined in all the samples collected.

### Animals and extraction of rumen fluid

Rumen fluid was obtained from the rumen of four Merino sheep housed in individual cages, fitted with rumen cannula and fed 1 kg alfalfa hay daily with free access to water and mineral/vitamin licks. A sample of rumen content was collected before the morning meal in thermos-flasks, taken immediately to the laboratory, strained through several layers of cheesecloth and kept at 39ºC under a $CO_2$ atmosphere.

### *In vitro* dry matter digestibility

For the determination of the *in vitro* dry matter digestibility two different gravimetric techniques were followed: that described by Tilley and Terry (1963) and that proposed by Goering and Van Soest (1970). After 48 h of incubation in buffered rumen fluid, samples were subject to either a 48 h pepsin-HCl digestion as described by Tilley and Terry (1963) or gently rinsed in cold water and treated with a neutral detergent solution at 100ºC for 1 h as described by Goering and Van Soest (1970). The residue remaining after drying was used to calculate the *in vitro* dry matter digestibility.

### *In vitro* gas production

The method used for the gas production measurements was as described by Theodorou *et al*. (1994). Incubations were carried out in serum bottles, and the volume of gas produced in each bottle was recorded at different incubation times, using a pressure transducer. Gas production from the samples was corrected by subtracting the volume of gas produced from blank cultures. An exponential model was fitted to the data to estimate fermentation kinetic parameters such as the asymptotic gas production or the fractional fermentation rate.

### Measurement of phenolics and tannins

Samples (200 mg dry weight) were weighed into glass tubes. The pigments were removed by a double extraction with 10 ml of diethyl ether containing 1% glacial acetic acid, including ultrasonication at room temperature for 5 min and centrifugation at 3000 g for 10 min (20ºC). The supernatant was decanted and the solid residue was dried for 2 h at 50ºC. Phenolic compounds and tannins were extracted from the solid residue by adding 15 ml of 70% aqueous acetone and gassing the headspace with $N_2$, followed by a gentle stirring for 15 min, sonication for 20 min at 4ºC and finally centrifugation (3000g x 10 min, at 4ºC). The supernatant was stored for analysis at 4ºC. Total extractable phenols (TEP) in the extracts were determined according to the method of Julkunen-Tiitto (1985) by using Folin-Ciocalteau and $Na_2CO_3$ (20%) as reagents. Tannic acid was used as the standard, and the absorbance was read at 725 nm using a Kontron Spectrophotometer (Uvikon 940).

The method designed by Makkar *et al*. (1993) was used for the indirect determination of total extractable tannins (TET) as the difference of TEP before and after tannin precipitation from the tannin-containing extract with insoluble polyvinylpyrrolidone (PVP). The reagents and the standard were the same used previously in the determination of TEP. The extractable condensed tannins concentration was determined according to the butanol method (CTb) of Porter *et al*. (1986), using the butanol-HCl reagent and ferric ammonium sulphate. The standard used was a solution of purified quebracho tannin. Absorbance was read at 550 nm in Biokinetics ELISA-microplates reader (Cultek TL 340). The CT were also determined following the vanillin assay (CTv) of Broadhurst and Jones (1978), using vanillin and concentrated HCl as reagents, and a solution of catechin as standard. Absorbance was read against a blank at 500 nm.

### Biological activity of tannins

The biological activity of tannins was assessed following the methods described by Khazaal *et al*. (1994) and Makkar *et al*. (1995). PEG is a tannin-binding agent, and hence the addition of PEG to the incubation medium may neutralize tannins resulting in an increase in either the dry matter digestibility or the fermentation gas production *in vitro*. Based on this principle, several *in vitro* trials were carried out, measuring dry matter (DM) disappearance or gas production at 24 or 48 h of incubation with and without PEG. Cultures without PEG were considered as control treatments.

### Data and statistical analysis

The effect of PEG on *in vitro* DM digestibility and gas production was calculated as the difference between values with and without PEG, as percentage of the control values. The statistical significance of the difference between mean values with and without PEG was assessed with a *t*-test. A simple correlation analysis was used to establish the relationship between techniques. Statistical analyses were performed with SAS software (SAS Institute, Cary, NC).

## Results and discussion

*In vitro* gas production kinetics and digestibility values followed the same trend (Fig. 1) and ranked the forages in the same order, with highly significant ($P<0.001$) and positive correlation coefficients between digestibility and all gas production parameters, except the gas production rate (c) that was not significantly correlated with the IVD determined by the Tilley and Terry technique (Table 1).

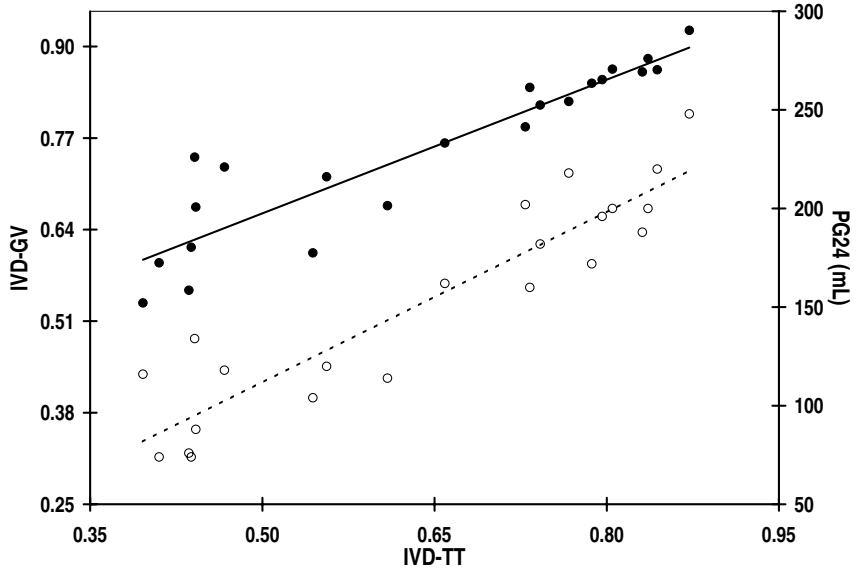


Fig. 1. Relationship between *in vitro* digestibility (IVD-TT and IVD-GV, solid symbols) and gas production (PG24, open symbols).

Table 1. Correlation coefficients between *in vitro* digestibility, gas production and fermentation rate

| | IVD-TT | IVD-GV | PG24 | c |
|---|---|---|---|---|
| IVD-TT | 1.000 | 0.933** | 0.929** | 0.403 |
| IVD-GV | | 1.000 | 0.890** | 0.468* |
| PG24 | | | 1.000 | 0.595* |
| c | | | | 1.000 |

IVD-TT: *in vitro* digestibility (Tilley and Terry); IVD-GV: *in vitro* digestibility (Goering and Van Soest); PG24: *in vitro* gas production at 24 h of incubation; c: fractional fermentation rate.
*P<0.05; **P<0.01; ***P<0.001.

According to Van Soest (1994), the original method of Tilley and Terry (1963) is a measurement of the apparent *in vitro* digestibility, whereas the extraction with the neutral detergent removes bacterial cell walls and other endogenous products, and therefore predicts true digestibility. As expected both measures were strongly correlated. The *in vitro* gas production technique has some advantages over gravimetric *in vitro* methods. It has been used to estimate digestibility (Menke and Steingass, 1988) and to assess the biological activity of tannins and other anti-nutritional factors on the digestibility of browse plants (Khazaal *et al*., 1994).

Across the different plant species, the *in vitro* DM digestibility and the volume of gas measured at 24 h of incubation showed significant correlations with the chemical composition of the browse foliage, positive with the CP concentration and negative with the fiber fractions (Kaitho *et al*., 1998), especially with the ADF and lignin contents (Table 2). The poor and non significant correlation (P>0.05) observed between c and the other studied parameters suggests that fermentation rate in the rumen is not only determined by the chemical composition of the forage, and many other factors (plant cell wall structure and composition, rumen microbial environment) may influence the degradation rate of forages in the rumen (López *et al*., 1998).

Table 2. Correlation coefficients between *in vitro* digestibility and chemical composition of the browse plants

| | IVD-TT | IVD-GV | PG24 | c |
|---|---|---|---|---|
| CP | +0.784* | +0.660*** | +0.807*** | +0.471* |
| NDF | -0.240 | -0.492* | -0.230 | -0.187 |
| ADF | -0.659*** | -0.791*** | -0.603** | -0.158 |
| Lignin | -0.701*** | -0.720*** | -0.640** | -0.058 |
| ET | -0.342 | -0.307 | -0.448* | -0.155 |
| CTv | -0.779*** | -0.519* | -0.617** | +0.078 |
| CTb | -0.749*** | -0.648** | -0.708*** | -0.083 |
| G24 | -0.817*** | -0.661*** | -0.835*** | -0.287 |
| G48 | -0.836*** | -0.675*** | -0.817*** | -0.177 |

IVD-TT: *in vitro* digestibility (Tilley and Terry); IVD-GV: *in vitro* digestibility (Goering and Van Soest); PG24: *in vitro* gas production at 24 h incubation; c: fractional fermentation rate; CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; ET: extractable tannins; CTv: extractable condensed tannins measured with the vanillin assay; CTb: extractable condensed tannins measured with the butanol-HCl assay; G24 and G48: effect of PEG (increase as % of control) on volume of gas produced after 24 and 48 h of incubation.
*P<0.05; **P<0.01; ***P<0.001.

Lignin can be assumed completely indigestible and represents a physical barrier limiting the access of microbial enzymes to the cell wall (Van Soest, 1994). The composition and structure of the cell wall may affect digestibility to a greater extent than the cell wall content, thus the higher negative correlations of digestibility with ADF or lignin contents than with the NDF content. Tannins can also affect cell wall digestibility to a variable extent because the interactions between tannins and cell wall carbohydrates vary with plant species, animal species, tannin levels and possibly tannin structure.

As expected, digestibility and tannin concentrations were negatively correlated, and both IVD and G24 showed significant ($P<0.01$) and inverse linear relationships with either the CT concentrations or the tannin biological activity assessed using PEG (Fig. 2). Recently, there is a renewed interest in the use of the gas production technique combined with the use of PEG as a useful tool for screening the biological activity of anti-nutritive factors of tanniniferous feeds on rumen fermentation. The high affinity of PEG by tannins prevent the formation of potentially indigestible tannin-protein complexes. Based on this principle, the specifics effects of tannins on digestion processes can be investigated, as the extent of PEG binding is a measure of the total amount of tannins in the sample (Jones *et al.*, 2000) and their ability to affect negatively the degradation of the feed in the rumen and its digestibility. The relative increase in gas production as consequence of the addition of the tannin-complexing agent represents the quantitative effect of tannins; the higher the biological activity of tannins on rumen microbes, the higher the increase in gas production as result of the neutralization of tannins by PEG. If the activity of condensed tannins in all shrubs was the same then a positive linear increase in gas production due to PEG would be expected as the level of condensed tannins was higher. The main advantages of this technique over the spectrophotometric methods are that biological activity rather than concentration is determined and that is not dependent upon a standard.

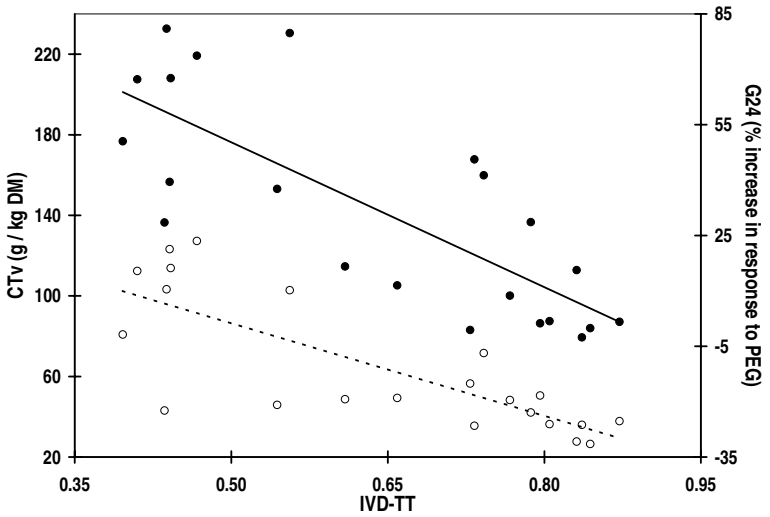


Fig. 2. Relationship between in vitro digestibility (IVD-TT) and tannin content measured either by chemical (CTv, open symbols) or by biological (G24, solid symbols) assays.

## Conclusions

Lignin incrustation of plant cell wall associated with a possible presence of phenolic compounds, such as tannins, are probably the most important factors which can potentially limit cell wall and forage digestibility, particularly at mature stages. Our results confirm that the *in vitro* gas production technique coupled with PEG binding appears to have a promising potential prospect for the assessment of phenolic-related anti-nutritive effects in feeds.

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