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Correlation between analytical and biological techniques for the assessment of phenolic compounds in Spanish browse species

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RESUME – *"Corrélation entre techniques analytiques et biologiques pour l'évaluation des composés phénoliques chez deux espèces arbustives espagnoles".·Vingt deux échantillons (feuilles et fleurs) collectés de six espèces arbustives Méditerranéennes ont été analysés pour leur contenus en phénols totaux extraites (PTE), tanins extraites (TE) et tanins condensés en utilisant soit la méthode de butanol-HCl (TCE-but) ou celle de vanilline acide (ECT-van). L´activité biologique des composés phénoliques, déterminée comme l´effet du polyethylène glycol 6000 (PEG 6000) sur la digestibilité* in vitro *(DIV) et la production de gaz mesurée après 24 et 48 h d´incubation dans le jus de rumen, a été étudiée. Une faible corrélation a été observée entre PTE et TCE-but/TCE-van. Cependant, des coefficients de corrélation significatifs et élevés ont été trouvés entre PTE et TE, d´une part, et entre TEC-but et TCE-van d´autre part. Il en est de même, une corrélation positive et significative (P<0,01) a été établie entre les tanins et leur activité biologique mesurée comme l´effet du PEG 6000 sur la production de gaz.*

Mots clés : *Plantes arbustives, tanins, production du gaz* in vitro*, polyéthylène glycol.*

Introduction

Most browse species contain variable amounts of structurally diverse secondary compounds such as phenolics, tannins and other compounds with anti-nutritional properties. In the process of evaluating the feeding value of this sort of fodder, the quantification of tannins seems to be an important issue. Actually, a growing number of methods are available for analysis of tannins; although these assays, due to the complex and diverse nature of tannins, do not provide satisfactory results. For a quick assessment and initial screening of tannins in browse species, it would be cheaper if tannins could be predicted from the use of relatively simple methods such as a tannin bioassay. This assay is based on *in vitro* rumen fermentation system coupled with the use of a tannin-complexing agent, polyethylene glycol 6000 (PEG 6000). However, there is little information about the relationship between the analytical and the biological methods for tannins. This information is essential to improve the accuracy of the determination of tannins in browse species and to understand the nutritional and metabolic significance of condensed tannins. The objective of this study was to assess the correlation between the concentration of phenolic compounds determined by different analytical techniques and the biological activity of tannins measured as the effect of PEG on *in vitro* dry matter digestibility (IVD) or gas production of 22 Mediterranean browsing samples.

Material and methods

Source of shrubby samples: twenty two samples consisting of leaves and flowers from six browse species were collected at different maturity stages from spring to autumn in 1998. 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 in the studied zone, uplands of the province of León (Norwest of Spain). The browse plants were clipped with scissors harvesting a mixture of leaves and fine green stems (Ø < 5 mm) or flowers. In the laboratory, leaves and flowers were separated by hand from the original samples, immediately freeze-dried and milled in a hammer mill using a 1-mm sieve for later analysis.

Animals and extraction of rumen fluid: rumen fluid was obtained from four rumen cannulated Merino sheep fed 1 kg alfalfa hay daily. A sample of rumen contents was collected before the morning

meal in thermos-flasks and taken immediately to the laboratory where was strained through four layers of cheesecloth and kept at 39ºC under a CO_2 atmosphere.

In vitro dry matter digestibility: In vitro dry matter digestibility was carried out following the technique proposed by Goering and Van Soest (1970). Rumen fluid was diluted into the culture medium in the proportion 1:4 (v/v). Samples (250 mg) were weighed out into polyester bags and incubated for 48 h in diluted rumen fluid, followed by an extraction in neutral detergent solution at 100ºC for 1 h. The incubation was carried with or without the addition of 10 g PEG (molecular weight 6000) per litter of the culture medium. Incubation runs were conducted in three consecutive weeks (three replicates per treatment).

In vitro gas production: About 300 mg of each sample were weighed out into serum bottles kept at approximately 39ºC and flushed with CO_2 before use. Two bottles were used for each substrate in each incubation run, one with 10 mg PEG/ml and the other with no PEG (control). Fifty ml of rumen/buffer mixture was anaerobically dispensed in each bottle. All the bottles were crimped and placed in the incubator at 39ºC, being shaken at regular times. The volume of gas produced in each bottle was recorded at 24 and 48 h after inoculation time, using a pressure transducer (Theodorou *et al*., 1994). Three runs of incubations were performed in different weeks (three replicates per treatment).

Phenolic compounds: After removal of pigments and lipids, phenolic compounds and tannins were extracted from the samples with 70% aqueous acetone following the steps described by Makkar (2000). Total extractable phenols (TEP) were determined in the extracts according to the method of Julkunen-Tiitto (1985), using Folin-Ciocalteau and Na_2CO_3 (20%) as reagents and tannic acid as the standard. Extractable tannins (ET) were estimated indirectly after being adsorbed to insoluble polyvinylpyrrolidone (PVP) and measuring the remaining total phenols in the supernatant as described by Makkar *et al*. (1993). Extractable condensed tannins were measured using either the butanol-HCl assay (ECT-but) reported by Porter *et al*. (1986) with the modifications of Makkar (2000) or the vanillin assay (ECT-van) of Broadhurst and Jones (1978). For ECT-but a solution of purified quebracho tannin was used as standard, whereas for ECT-van the standard was a solution of catechin. Concentrations of all phenolic compounds were expressed in g/kg of DM, standard equivalent.

Data and statistical analysis: Effects of PEG either on IVD or on gas production were calculated as the difference between values with and without PEG, as percentage of the value observed in the control. A simple correlation analysis was used to establish the relationship between techniques.

Results and discussion

As shown in table 1, browsing samples could be split into a group with high concentration of phenolics and another with a generally low phenolics content. Such variations should be attributed mainly to the differences in browse species, maturity stage and morphological part (leaves or flowers). ECT-but displayed the largest variation, corresponding the lowest value to young leaves of *C. scoparius* and the highest to mature leaves of *E. australis* (Ammar, 2002).

Table 1. Content of phenolic compounds in 22 Mediterranean browsing samples (leaves and flours) determined by different analytical techniques

Phenolic component	Range		Mean
	min	max	
TEP (g/kg DM, acid tannic equivalent)	18.2	148.0	92.5
ET (g/kg DM, acid tannic equivalent)	3.4	130.5	75.3
ECT-but (g/kg DM, quebracho equivalent)	3.2	899.5	201.2
ECT-van (g/kg DM, catechin equivalent)	26.5	127.3	64.5

Moreover, results stated herein displayed a considerable variation related to the method of analysis. Similar results were also reported in other Mediterranean browse species (Khazaal *et al*., 1994). This variation is expected since the chemical proprieties that are involved in the reactivity of

polyphenols determined by the different methods are widely different. A large difference was observed between the two techniques used in the extraction of condensed tannins (Table 1). The vanillin assay is unspecific since other flavanoids and not only tannins can react with vanillin (Makkar, 2001) and absorbency intensifies as molecular size decreases. Moreover, the standard used (catechin) has no tannin properties and CT would be overestimated. However, the butanol-HCl method is based on the formation of cyanidin from the depolymerisation of the tannin molecule; therefore, it determines the number of monomers irrespectively of their biological activity. Besides, quebracho tannin used in this technique is characterized by a low reactivity (Makkar, 2001) which would probably lead to a significant overestimation of CT in the samples. The deficiencies in the butanol-HCl technique mentioned here can explain partly the unrealistic concentrations of CT in some browsing samples. For example, it is not possible that the CT content reach approximately 900 g/kg of DM (Table 1). It seems also very unlikely that CT concentration to be higher than TEP concentration, as there are many other phenolic compounds different from condensed tannins. Therefore, this method should be used with caution as a quantitative assay. The difference between the ET values obtained by the procedure of Makkar *et al*. (1993) and the ECT-but is due to different principles of the reaction of each method and different standards used (Makkar, 2001). On the other hand, the Folin Ciocalteau reagent is specific for phenolic compounds but it does not discriminate between tannin and non-tannin phenolics. As depicted in Table 2, butanol-HCl and vanillin methods were strongly correlated ($r = 0.758$, $P < 0.001$). High and significant correlations between these two techniques were reported previously by Khazaal *et al*. (1994). This suggest that samples were ranked in the same order using one or another technique. In this respect, Makkar (2001) suggested that butanol-HCl is the preferred method for analysis of CT in feedstuffs. The poor correlations between the other analytical assays suggests that no single method will give results that are satisfactorily related to nutritional effects and therefore a greater understanding of the types and amount of tannins present in browse species is needed, together with the development of methods aimed to correctly quantifying biologically "active" tannins (Schofield *et al*., 2001).

Table 2. Correlation coefficients (r) between concentration of total extractable phenols (TEP), extractable tannins (ET), extractable condensed tannins (butanol-HCl -ECT-but- and vanillin –ECT-van-), and the effects of polyethylene glycol on *in vitro* dry matter digestibility (IVD) or gas production (G24 and G48) of 22 Mediterranean browsing samples

	TEP	ET	ECT-but	ECT-van	DVS
TEP	1.000	0.973	0.428	0.241	
ET		1.000	0.459	0.324	
ECT-but			1.000	0.758	
ECT-van				1.000	
G24	0.731	0.780	0.821	0.812	0.386
G48	0.650	0.718	0.851	0.864	0.287
IVD	0.395	0.453	0.107	-0.170	1.000

N=22; $P<0.05$ if $|r| > 0.423$; $P<0.01$ if $|r| > 0.537$; $P<0.001$ if $|r| > 0.652$.

The *in vitro* gas method combined with the use of PEG 6000 is expected to be better than chemical methods for quantification of anti-nutritional effects. The relative increase in gas production represents the quantitative effect of tannins; the higher the biological activity of tannins on rumen microbes, the higher the increase in gas production in presence of PEG. Tannin content and their biological activity showed a negative correlation with the *in vitro* digestibility of browse species (Ammar, 2002), what is consistent with the established fact that phenolic compounds reduce digestibility and nutrient availability of feedstuffs (Makkar, 2001). The highest correlations ($r > 0.8$, $P < 0.001$) were found between CT tannins and the effect of PEG 6000 on gas production measured at 24 and 48 h (Table 2). This result suggests that the gas production technique could be complementary to some analytical methods.

However, the effect of PEG 6000 on IVD was not related ($P > 0.05$) to the concentrations of TEP, ECT-but and ECT-van (Table 2). At this stage, the lack of relationship between the increase in IVD and the phenolic concentrations could be due to the formation of insoluble tannin-PEG complexes, present as artifacts in the truly undigested residue. These results are in agreement with those reported by other authors (Makkar, 2001) and prove the convenience of the gas production technique

in studying the biological activity of tannins and predicting more accurately their adverse effects on rumen fermentation.

Conclusions

Analytical assays result in low accuracy and large variability of results owing the range of different principles of the reaction of each method and the use of different standards. Therefore, no single method will give results that are satisfactorily related to nutritional effects. The *in vitro* gas production technique combined with PEG 6000 appears to have a promising potential prospect for the assessment of phenolic-related anti-nutritive effects in feeds. However, the effects of tannins on *in vitro* digestibility using a gravimetrical should be interpreted with caution.

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