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Tannin levels in the foliage of some Spanish shrub species at different stages of development

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SUMMARY – Total extractable phenols (TEP), total extractable tannins (TET) and extractable condensed tannins (CT) were determined in the foliage of four Spanish indigenous shrub species namely *Erica australis* (Spanish heather), *Cistus laurifolius* (laurel-leaved rock-rose), *Quercus pyrenaica* (hoary oak) and *Rosa canina* (wild dog rose) obtained from uplands in the province of León (North West Spain). To follow seasonal variations within each species, leaves were collected at different times of the year, so that plants were at different stages of development. According to their contents in extractable CT, the shrub species were ranked in the order *E. australis* > *C. laurifolius* > *R. canina* = *Q. pyrenaica*. In relation to the seasonal variations, extractable CT concentration was highest in summer for all species, except for *E. australis*, which showed the maximum content in autumn. Young leaves of *Q. pyrenaica* and *C. laurifolius* collected in spring had higher TEP and TET contents than those collected in autumn, whereas the opposite trend was observed for *R. canina* leaves and little seasonal differences in TEP and TET concentrations were detected in *E. australis*. Leaves of all four species had high concentrations of these secondary compounds in all seasons, which may be an important constraint for the use of these plants by ruminants browsing upland areas where these shrub species are predominant.

Key words: Tannins, phenols, browse plants, shrubs.

RESUME – *"Variations saisonnières des teneurs en tannins du feuillage de quelques arbustes espagnols". Les teneurs en phénols totaux libres (PTE), tannins totaux libres (TTE) et tannins condensés libres (TCE) du feuillage de quatre arbustes (*Erica australis*,* Cistus laurifolius*,* Quercus pyrenaica *et* Rosa canina*) ont été déterminées. Des échantillons de chaque espèce ont été prélevés de la montagne de la province de León (Nord-Ouest de l'Espagne) au printemps, en été et en automne. Le classement des arbustes, par ordre décroissant selon leurs teneurs en TCE, a été le suivant :* E. australis > C. laurifolius > R. canina = Q. pyrenaica*. La concentration des TCE libres de toutes les espèces a atteint le maximum en été, sauf dans le cas de* E. australis *qui a montré le maximum en automne. Les jeunes feuilles de* Q. pyrenaica *et de* C. laurifolius *collectées au printemps ont des teneurs en PTE et TTE plus élevées que celles des feuilles collectées en automne. En revanche, une tendance opposée a été observée pour les feuilles de* R. canina *et de très légères variations saisonnières des teneurs en PTE et TTE ont été détectées au niveau de* E. australis*. Les feuilles des quatre espèces arbustives étudiées sont riches en composés secondaires tout le long de l'année. Ceci pourrait limiter le potentiel fourrager de ces arbustes, en particulier leur utilisation par les ruminants sur parcours.*

Mots-clés : *Tannins, phénols, espèces arbustives.*

Introduction

In arid regions with a pronounced dry season and in mountain areas and highlands with cold winters, there may be periods during the year with a serious shortage of conventional feedstuffs for the animal. During these critical periods when grasses and forbs are dormant, browse species supply green material for grazing animals, thus being the only source of nutrients. However, there are a number of constraints for the use of browse plants as animal feeds. They are often inaccessible to grazing animals, their foliage has higher fibre and lignin contents than grasses, and also higher levels of soluble phenolic/tannin compounds than herbaceous species (Lefroy *et al*., 1992). Recommendations about the use of tanniniferous forages are complicated owing to the effect of the maturity stage on the tannin concentration. The knowledge of this effect is essential to achieve an optimum use of these resources as forage for ruminants.

The main objective of the present study was to determine the contents of phenols and tannins (extractable and condensed tannins) in the foliage of some Spanish shrub at different maturity stages using different methods for analysis.

Material and methods

Leaves were harvested from four Spanish indigenous species widespread in uplands of the province of León (North West of Spain). The sampling area is at an altitude of 900 m, and climate is characterised by a mean rainfall of 564 mm per year and an average temperature of 10.6°C. The browse plants were: (i) *Erica australis* (Spanish heather); (ii) *Cistus laurifolius* (laurel-leaved rock-rose); (iii) *Quercus pyrenaica* (hoary oak); and (iv) *Rosa canina* (wild dog rose). These species were selected as the most representative of those eaten by grazing sheep and goats during critical periods of grass shortage. Sampling took place at three different times in 1998, namely: (i) May-June (spring); (ii) July-August (summer); and (iii) October-November (autumn); so that plants were at different maturity stages. The browse plants were clipped with scissors collecting a mixture of leaves and thin stems. In the laboratory, leaves were manually separated from the original samples. The leaves were then immediately freeze-dried and ground in a hammer mill using a 1-mm sieve. 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 (20°C). The supernatant was decanted and the solid residue was dried for 2 h at 50°C. 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 4°C and finally centrifugation (3000 g $\times$ 10 min, at 4°C). The supernatant was stored for analysis at 4°C. 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 (CT) concentration was determined according to the method of Porter *et al*. (1986), with the modifications of Makkar (2000), 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).

Results and discussion

There were considerable differences in the contents of phenolic compounds among species, and among sampling seasons within each species. The concentration of these secondary compounds may change as plants mature because of the physiological changes occurring during the plant growing cycle. Soil type, fertility and water supply are known to affect tannin levels in plants. Moreover, species vary in their response to climatic and physiological changes (Dann and Low, 1988), that induce changes in the chemical composition and, in particular, in the concentration of secondary compounds like tannins. These differences determine the value of browse plants foliage as a forage resource for ruminants.

The seasonal variations of TEP in leaves of the four shrub species are presented in Fig. 1. For *C. laurifolius* and *Q. pyrenaica*, TEP content was higher in young leaves and decreased as plants matured. The observed changes in TEP associated with the maturation process are in accordance with the findings reported for some oak species by Makkar *et al*. (1988, 1991). However, the opposite trend was observed for *R. canina* and *E. australis*, although the differences among sampling seasons were small. Irrespective of the maturity stage, *E. australis* and *R. canina* showed the highest TEP contents.

The changes in TET contents (Fig. 2) followed a similar trend to those observed for TEP, as TET represented on average 90% of the TEP (ranging from 83 to 94% depending upon the browse species and the sampling time), suggesting that only a small proportion of TEP remained as non-precipitable phenols. In all species, except in *E. australis*, this percentage was higher in young leaves and decreased slightly with maturity. It is noteworthy that the proportion of TEP that are recovered as TET was significantly lower with other indigenous browse species, like *Genista florida* or *Cytisus scoparius* (data not shown).

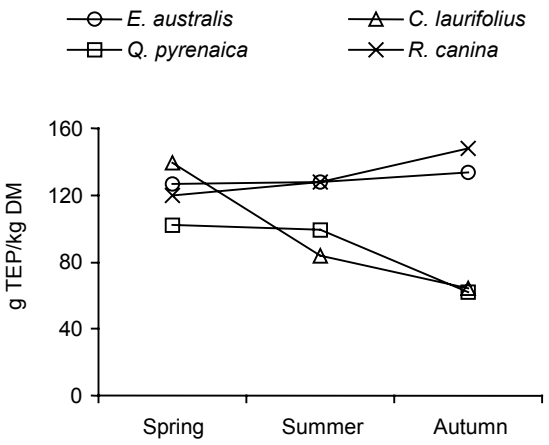

Fig. 1. Seasonal variations in total extractable phenols (TEP) concentrations in the foliage of the browse plants [as tannic acid equivalents, g/kg dry matter (DM)].

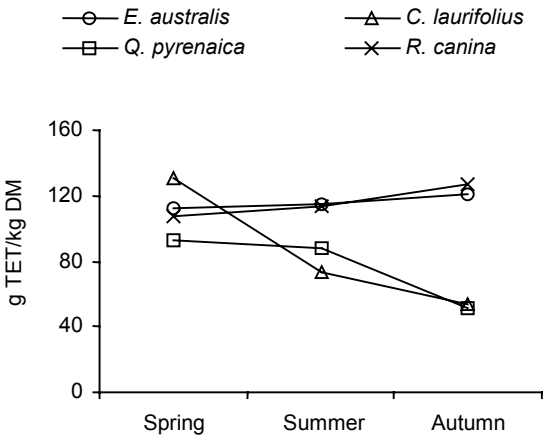

Fig. 2. Seasonal variations in total extractable tannins (TET) concentrations in the foliage of the browse plants [as tannic acid equivalents, g/kg dry matter (DM)].

Seasonal variations of TET followed different patterns in each plant species. There was a steady fall with maturity in the TET concentration of *Q. pyrenaica* and *C. laurifolius* foliage. This was consistent with the findings of Hagerman (1988). On the contrary, TET concentrations of *E. australis* and *R. canina* foliage were slightly higher in mature than in young leaves. Seasonal differences in tannin contents may have been related to the phenology of each plant species and to the temperatures prevailing during the different stages of growth.

Contents of extractable CT in shrub leaves and the fluctuations among seasons are depicted in Fig. 3. *E. australis* showed a high content in extractable CT, according to the findings of Tolera *et al*. (1997) who showed the high levels of CT in the foliage of *Erica* sp. In all studied species, extractable CT formed a large part of the total CT, including protein- and fibre-bound CT (data not shown). Young leaves of *Q. pyrenaica* and *R. canina* showed a low concentration of extractable CT, in agreement with our earlier findings where *in vitro* gas production (Ammar *et al*., 2000) and *in vitro* dry matter digestibility (Ammar *et al*., 2001) of the foliage of these species (determined by incubation in sheep rumen liquor) showed no response to the addition of PEG 6000 (indicator of the biological activity of tannins). On the other hand, except *E. australis*, all species showed the maximum of extractable CT in summer. This result was in agreement with the results of Cabiddu *et al*. (2000) about the CT content of various browse species, and could reflect the effect of the high temperatures on the level of tannins.

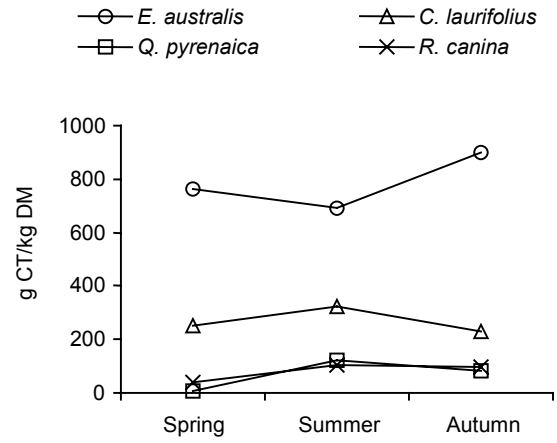

Fig. 3. Seasonal variations in extractable condensed tannins (CT) concentrations in the foliage of the browse plants (as quebracho tannin equivalents, g/kg DM).

It is noteworthy that some of the CT concentrations measured cannot be considered realistic. For instance, it is not possible that the CT content in *E. australis* foliage is over 700 g/kg dry matter at all sampling seasons. It seems also very unlikely CT concentration to be higher than TEP concentration, as there are many other phenolic compounds different from condensed tannins. These unrealistic results can be due, in part, to deficiencies in the butanol-HCl technique to measure condensed tannins (Giner-Chavez *et al*., 1997; Schofield *et al*., 2001). The main drawback of this method is the lack of a reliable standard to estimate CT concentrations from the absorbance values. Purified quebracho tannin has been questioned as standard because of its globular structure and the small number of hydroxyl groups that are responsible for a low reactivity of the molecule, resulting in low absorbance values even with high concentrations of quebracho tannin (Schofield *et al*., 2001), thus overestimating the concentrations of CT in the samples when calculated from the standard curve obtained with quebracho tannin. Makkar (2000) has suggested to use only the absorbance values or to apply a conversion factor to estimate CT concentrations as leucocyanidin equivalent from absorbance values. Although this may give values that could be considered more realistic, some authors have raised the possibility that the reaction with the butanol-HCl-iron reagents may be specific for each tannin, depending on the number and type of reactive groups present in its molecule. In this case, the colour development as result of the reaction would be different for each plant species and the use of absorbance values or concentrations estimated from a single external standard would be of limited use for the comparison among species that may contain tannins of different chemical structure. Only concentrations estimated from the so called "internal standards" (i.e. using for each species tannins isolated and purified from plants of the same species) could be considered as the best approximations to the real values (Schofield *et al*., 2001). Some authors have observed that with this approach the concentrations are more realistic, and the values follow similar trends to those observed with the TEP and TET concentrations determined by using the Folin-Ciocalteau technique (M. Alvarez and P. Frutos, pers. comm.). The butanol-HCl reaction should be used with caution as a quantitative assay due to the heterogeneity of CT and the lack of appropriate standards for their quantification. Although the use of "internal standards" could overcome the main drawback of the determination of CT in shrub and tree foliage, the isolation and purification of CT from each species are expensive and time consuming, and thus cannot be accepted as routine procedures for most laboratories. Despite the shortcomings of the technique, the acid-butanol assay is the most commonly used for determination of CT in plants.

Due to the low correlation between the different methods used for tannin analysis, a greater understanding of the types and amount of tannins present in browse species is needed, together with the development of methods aimed at correctly quantifying biologically "active" tannins (Kaitho *et al*., 1998; Schofield *et al*., 2001). Only through such detailed studies can the influence of these compounds on the nutritional value of forages be accurately assessed.

Conclusion

This study highlights the considerable differences in the levels of phenolic compounds in the foliage of some Spanish browse plants in relation to the botanical species and their seasonal variations. The discrepancies between analytical techniques in the observed tannin levels suggest that some of these techniques are of limited use even for ranking and comparing different browse plant species, and raise the need for developing more reliable and precise techniques.

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