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TANNINS OF HERBACEOUS LEGUMINOSAE

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Abstract—The tannins of the leaves of five of the relatively few species of herbaceous Leguminosae which contain them, consist of leucocyanidin and leucodelphinidin. In most of the species (sainfoin, *Onobrychis viciaefolia* Scop. especially) the tannins are difficult to extract from the leaves. Methods, based on haemanalysis, have therefore been developed for determining the tannic acid equivalent and the relative astringency in leaf tissue finely divided by milling and sieving. The particular case of sainfoin, with its bearing on bloat in cattle, is considered in detail.

ALTHOUGH tannins are regularly present in the seedcoats of leguminous plants,[1] they are usually absent from the vegetative parts of herbaceous species. Exceptions are the members of the Hedysareae, conspicuously the fodder crops *Lespedeza cuneata* Don (sericea) and *Onobrychis viciaefolia* Scop. (sainfoin), and the following native British species: *Trifolium arvense* L., *Lotus corniculatus* L. and *Lathyrus pratensis* L., many of which are at present under investigation in New Zealand and elsewhere as possible forage species on account of their usefulness in the prevention of bloat in ruminant livestock.

All of these species contain leuco-anthocyanins (LA's) producing both cyanidin (Cy) and delphinidin (D) when heated with mineral acid; gallo- and ellagi-tannins are absent. It is not known whether the LA's are of the proanthocyanidin or leucoanthocyanidin type.[2] The former are condensation products of flavan-3-ols (catechins) and the latter molecular complexes of flavan-3,4-diols. For present purposes it will be assumed that they are of the former type since these appear to be the commonest.[3]

Typical procyanidins can be determined by spectrometric measurement of the cyanidin produced by heating under standard conditions in butanol containing hydrochloric acid.[4,5] Anthocyanidin production is not stoichiometrical, but after 2 hr approaches a steady value. The procyanidins previously examined,[4] which were dimers, trimers, tetramers and higher oligomers of catechin and epicatechin, had $E^{1\%}_{1cm}$ values ranging from 90 to 200 according to MW and in this paper an average value of $E = 150$ has been assumed in converting E values into per cent tannin. No prodelphinidins have been available for corresponding values to be obtained. The amount of tannin extracted by aqueous methanol from leaves of several of the above species reaches as much as 16%, estimates which are confirmed by the determination of the tannic acid equivalent of extracts of the leaves by haemanalysis.[4] Such extracts

[1] BATE-SMITH, E. C. and RIBÉREAU-GAYON, P. (1959) *Qual. Plant.* **5**, 189.
[2] WEINGES, K., BAHR, W., EBERT, W., GORITZ, K. and MARX, H.-D. (1969) *F. Chem. org. Naturstoffe* **27**, 158.
[3] THOMPSON, R. S., JACQUES, D., HASLAM, E. and TAYLOR, R. J. N. (1972) *J. Chem. Soc., Perkin Trans.* **1**, 1387.
[4] BATE-SMITH, E. C. (1973) *Phytochemistry* **12**, 907.
[5] SWAIN, T. and HILLIS, W. E. (1959) *J. Sci. Food Agri.* **10**, 63.

do not, however, contain the whole of the proanthocyanidins present in the leaves; in some cases, conspicuously in sainfoin, the amount extracted even under the most favourable conditions is only a small proportion of the total present in the fresh leaf as judged by direct analysis of the unextracted tissue. This fact seems to be associated with the presence of leucodelphinidin as a constituent of the tannin.

This situation has been studied in detail in sainfoin, because it is an important property in connection with the use of this plant for forage and in the control of bloat. The presence of leucodelphinidin seems also to be associated with the failure of anthocyanidin to be produced when intact leaf tissue is heated in butanolic hydrochloric acid. In such circumstances, the tissue becomes intensely dark but only a little colour is extracted into the butanol. If, however, the tissue is first briefly boiled in 50% aq. methanol, subsequent heating in BuOH–HCl turns it translucent and the solvent becomes intensely coloured. The *E* values recorded in Table 1 for leaf tissue refer to samples so treated.

RESULTS

Analysis of Leaf Tissue

The results of analysis of tissue and aqueous methanolic extracts of leaves of 5 species are shown in Table 1. *Lespedeza cuneata* was not available, but *Hedysarum multijugum* Maxim., in the same tribe, was examined as well as *Onobrychis viciaefolia*. The $E^{1\%}_{1cm}$ values and the tannic acid equivalent (TAE)[4] are all referred to dry wt of tissue.

TABLE 1. ANALYTICAL DATA FOR 5 SPECIES OF LEGUMINOSAE

Species	*E* values of leaf tissue†	*E* values of leaf extract	Tannic acid equivalent of extract	Relative astringency of tannin*
Trifolium arvense L.	15·5	7·0	4·4	0·94
Lotus corniculatus L.	5·0	2·0	1·5	1·1
Hedysarum multijugum Maxim.	21·5	17·5	5·2	0·45
Onobrychis viciaefolia Scop.	21–24	3·0	1·5	0·75
Lathyrus pratensis L.	19·5–22·5	24·0	11	0·7

* Assuming $E^{1\%}_{1cm}$ of tannin = 150.
† First treated with 50% MeOH at 100° for 5 min prior to analysis (see Experimental).

Analysis of Milled Leaf

When a larger sample of sainfoin was milled, the finely divided (100 mesh) sample had the same *E* value as obtained with the whole leaf, but the aqueous methanol extract now had $E = 6{\cdot}0$, twice that of the unmilled leaf (Table 1). Milling had, therefore, either produced a fraction richer in LA, or fine division had improved its extractability. The ability of the milled samples *as such* to precipitate blood protein was next examined. Weighed amounts of milled leaf were suspended in appropriate volumes of 1:100 diluted blood, stirred vigorously for several minutes and centrifuged. The residual haemoglobin was measured spectrometrically, and the TAE calculated.[4] The TAE of the 100 mesh sample was 4·5, compared with 1·5 for the extract prepared from the unmilled leaf (Table 1). Fine milling is necessary to achieve this efficiency; a 60 mesh sample had TAE = 3·7, and the residue not passing 60 mesh, 2·75.

[6] BATE-SMITH, E. C. (1972) *Phytochemistry* **11**, 1153.

Effect of Maturity of Leaf

The sainfoin used for the above experiment was a mixed sample of the whole foliage cut in August, on the point of flowering. It seemed likely that the maturity of the leaf would affect its tannin content and a sample of the second growth of the season, cut in October was divided into young leaves (selected at the 'two leaves and a bud' stage from shoots not yet forming a flower) and mature leaves (a mixed sample of the remaining leaves of the cut).

TABLE 2. ANALYTICAL DATA FOR YOUNG AND MATURE LEAVES OF SAINFOIN. POWDERED LEAF PASSING 100 MESH SIEVE

	Solid			Extract		
Leaf	LA	TAE	RA	LA	TAE	RA
Young	21·9	8·8	0·40	18·3	12·0	0·65
Mature	19·3	4·2	0·22	4·0	2·0	0·5

Milled 100 mesh fractions of each sample were prepared and the LA, TAE and RA determined. Aqueous methanol extracts were made from 100 mesh powders by appropriate modifications of the routine procedure, concentrated *in vacuo*, rediluted with water, and LA, TAE and RA determined. The results are given in Table 2.

TABLE 3. EFFECT OF LEUCODELPHINIDIN ON THE EXTRACTABILITY OF TANNINS

	% LA of milled leaf	% LA of extract	Fraction extracted
Species containing some LD			
Potentilla anserina L.	5·5	1·0	0·18
Miconia magnificatriana (De Vriese)	7·3	2·0	0·27
Shorea leprosula Miq.	9·0	nil	nil
S. macrophylla P. S. Ashton	11·0	tr	tr
**Acorus calamus* L.	3·5	0·3	0·09
**Eichonoria crassipes* Solms.	3·75	tr	tr
**Iris pseudacorus* L.	4·9	2·6	0·53
Vicia faba L. (seed-coat, green)	5·6	0·4	0·07
Species containing LCy only			
Agrimonia eupatoria L.	9·8	3·1	0·32
Fragaria moschata Duchesne	4·1	1·9	0·46
Prunus laurocerasus L.	20·0	12·0	0·60
Sorbaria aitchisonii Hemsl.	21·5	11·0	0·51
Neillia longiracemosa Hemsl.	16·0	8·0	0·50
Spiraea thunbergii Sieb.	12·3	8·0	0·65
Cotoneaster horizontalis Decne.	14·5	8·0	0·55
Quercus robur L.	5·5	2·7	0·49
Camellia japonica L.			
va. 'Jupiter'	4·0	3·6	0·90
white var.	6·0	5·8	0·96

* Pieces of leaf tissue.

There is little difference between the total LA content of the young and mature leaves, but there is an immense difference in the extractability of the tannin in the two tissues, which was reflected to some extent in the lesser ability of the milled mature leaf to precipitate protein.

That fine division is largely responsible for the ease with which the tannin is extracted from the young leaf is indicated by results which were obtained with residue not passing 100 mesh: only 3·3% of LA was present in the extract compared with 18·3% in that of the 100 mesh fraction. The corresponding mature leaf extract had only 0·9%.

No explanation can be suggested at present for this exceptional behaviour of sainfoin. It is not due solely to the presence of LD because prodelphinidins are present (as shown by PC) in all five species examined; nor to a relatively large proportion of LD, because *H. multijugum* has an even larger proportion. But LD does have an effect as shown by the results in Table 3, and it seems probable that this is mainly responsible for the sanfoin results. It must be also concluded that there may be a particular form of association between the tannin and other structural elements of the sanfoin leaf, but whether this is due to particular characteristics of the tannin or of those elements must await further investigation.

The observation that fine division facilitates the release of tannin does, however, support the conclusion reached by Mangan[7] that the action of the tannin takes place in the mouth as the forage is being masticated, and probably accounts for the almost complete absence of soluble protein in the bolus.

EXPERIMENTAL

Plant material. Except for the 2nd cut of sainfoin, the plants were collected in July and August when in flower. *H. multijugum* was supplied by the Cambridge University Botanic Garden. The leaves were oven-dried 24 hr at 40°.

Preparation of extracts. Leaf material was first boiled 5 min with 50% MeOH, decanted, macerated with sand and further quantities of 50% MeOH, and filtered through cotton wool. The combined extracts (centrifuged if necessary), were reduced to a small bulk *in vacuo* at 40°, and the residue taken up in the minimum vol. of H_2O.

Determination of LA. (A) *Of extract,* as described previously.[4] (B) *Of solid,* 2–5 mg of tissue, or of milled leaf, are heated for 5 min at 90–5° with 1 ml of 50% aq. MeOH, made up to 3·5 ml with 5% conc. HCl in BuOH, and heated for 2 hr. The tissue should become quite translucent, otherwise the extraction of anthocyanidin is incomplete and the result unreliable.

Determination of TAE. (A) *Of extract,* as described previously.[4] (B) *Of solid,* depending on the amount of tannin to be expected, 20–50 mg of 100 mesh milled leaf are treated, with vigorous stirring, with successive 1 ml vols of 1:100 diluted blood until an excess of haemoglubin is visibly present. The suspension is centrifuged and the absorptivity of the supernatant at 578 nm compared with that of a control. A residual absorption between 0·3 and 0·7 should be aimed at. The equivalent tannic acid concentration of the suspension = 0·015% + 0·024x% (x = fraction of haemoglobin precipitated) and from this the TAE of the sample can be calculated.[4]

RA. This is an attempt to represent the astringency of the tannin present relative to that of tannic acid, weight for weight, but it depends on an estimate being available of the *amount* of tannin in the sample. In the case of LA tannins, the only information on which such an estimate can be formed is the absorptivity of the anthocyanidin produced from it, together with the E values of the particular species of LA present. The value $E = 150$ assumed here is based on data published elsewhere,[4] but these data were only for procyanidins; corresponding data for prodelphinidins are not yet available. The values for RA in Table 1 and for LA and RA in Table 2 must therefore be regarded as provisional.

[7] MANGAN, J. L. (1968–9) *Bienn. Rep. Inst. Anim. Physiol. Babraham,* Babraham 1970, p. 96