Phytochemistry, 1976, Vol. 15, pp. 1407–1409. Pergamon Press. Printed in England.

THE CONDENSED TANNINS OF PASTURE LEGUME SPECIES

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(*Received* 5 *March* 1976)

Key Word Index—Leguminosae; condensed tannins; isolation; purification; molecular size distribution; prodelphinidin/procyanidin analysis; astringency.

Abstract—Condensed tannins have been isolated from legume pasture species and purified by gel chromatography on Sephadex G-50 and LH-20 media. Molecular size distribution, and composition (delphinidin/cyanidin ratio) have been determined for the pure polymers. The relevance of molecular size to extractability and astringency is discussed.

INTRODUCTION

Although condensed tannins (polymeric flavanols) have been recognised as secondary products in a variety of plants [1], including legumes, their chemical and physical properties have not been extensively studied owing to difficulties in isolation and characterization. Recent evidence has indicated that in some legumes, condensed tannins may have considerable agricultural importance in preventing bloat [2–4], and in protecting dietary proteins against microbial deamination in the rumen [5]. Both actions have been attributed to the ability of condensed tannins to form insoluble complexes with leaf and salivary proteins at rumen pH values [6] whereas at intestinal pH's the complexes were dissociated [7].

In order to study further the nature of the interactions between condensed tannins and proteins, it was considered necessary to first characterize the purified condensed tannins. This paper reports procedures for the isolation of condensed tannins in a pure form, their molecular weight distribution, and chemical composition.

RESULTS AND DISCUSSION

The condensed tannins, prepared by modifying earlier Sephadex separations [8, 9] (see Experimental), were white amorphous solids, completely soluble in water to give pale yellow solutions. Solutions of tannins and the dry tannins (freeze dried) gradually became brown on exposure to sunlight. The brown pigments, normally associated with tannin preparations, are either due to contaminants or artifacts produced by oxidation of the tannins.

In all the species examined, the polymeric fraction amounted to 90–99% of the total flavanols, the remainder being composed of catechins, gallocatechins and their dimers. The MW's of the tannins isolated in this work (Table 1) were larger than those reported earlier for the water soluble procyanidins [10]. The condensed tannins isolated from flowers of *Trifolium repens*, leaves of *Lotus pedunculatus* and *Trifolium arvense* were homogeneous in MW. Those isolated from the other species were heterogeneous. The most common MW's were between 7000–8000 though they varied in size from 5800 (*T. affine*) to 28000 (*Onobrychis viciifolia*).

Although several of the tannins were homogeneous in size, with the exception of *T. repens*, they were mixed flavanol polymers (Table 1). Treatment with *n*-butanol–HCl produced delphinidin and cyanidin, identified by standard procedures. It was not possible to determine whether the tannin occurred as a co-polymer of procyanidin and prodelphinidin or as separate procyanidin and prodelphinidin polymers or as a mixture of both. The composition of the polymers in terms of ratio of delphinidin to cyanidin did not vary within species, but was distinct for the different species.

Bate-Smith [11] has reported difficulty in extracting proanthocyanidins from sainfoin, *Onobrychis viciifolia* into aqueous methanol, due to its high prodelphinidin content, but noted that the proanthocyanidin from *Hedysarum multiguum*, although containing a high prodelphinidin content, was extractable with aqueous methanol. The exceptional behaviour of the sainfoin proanthocyanidin could be due to its very high MW (17000–28000). A correlation between polymer size and extractability into methanol, 50% aqueous methanol, and H_2O has previously been reported [10]. Aqueous acetone (3:7) can be used to extract >95% W/W of the

Table 1. MW distribution of condensed tannins

Plant species	Tissue	MW range of tannin	No. of monomers*	Dp/Cy† ratio
Onobrychis viciifolia Scop.	leaf	17000–28100	57–94	81:19
Coronilla varia L	leaf	10100–13200	34–44	71:29
Trifolium repens L.	flower	8500–9100	28–30	100:0
Trifolium arvense L.	leaf	7900–8200	26–27	57:43
Trifolium affine L.	leaf	5800–8700	19–29	47:53
Lotus pedunculatus Cav.	leaf	6800–7100	23–24	77:23

* Assuming a unit molecular weight of 300. † Dp = delphinidin, Cy = cyanidin.

proanthocyanidins in legumes, including sainfoin (W. T. Jones unpublished) and would appear to be a better solvent than aqueous methanol at least for extracting tannins of high prodelphinidin content.

Sainfoin is highly palatable to ruminants [5], whereas in other species, condensed tannins have been reported to be the causative agents that decrease palatability of the forage and give rise to low feed intakes [12]. With the exception of sainfoin tannin the astringency of the tannins was approximately in order of their prodelphinidin content, *T. affine* and *T. arvense* < *Lotus pedunculatus* < *Coronilla varia* < *T. repens*. Sainfoin tannin, although containing the highest level of prodelphinidin for the leaf tannins, was the least astringent. Low astringency of sainfoin may be explained by its high MW [10].

EXPERIMENTAL

Plant material Sainfoin, *Onobrychis viciifolia* Scop. var Fakir (ex I.N.R.A., Paris, France), var Melrose (ex Saskatoon Research Station, Saskatewan 37NOX2 Canada); Crownvetch, *Coronilla varia* L. var Chemung, var Pengift (ex Dr Cope, North Carolina State University, Raleigh, U.S.A.); Lotus major, *Lotus pedunculatus* Cav. var G4702, G4703, G4705 (ex Grassland Division, Palmerston North, New Zealand) and other selected ecotypes [13]; Haresfoot trefoil, *Trifolium arvense* L., Himatangi (M_3) local ecotype (ex W. T. Jones, Applied Biochemistry Division, DSIR, Palmerston North), N.Y.T. 411, N.Y.T. 1044 (ex volcani Centre, Neve Ya'ar, Israel), P.I. 244322 (Spain), P.I. 204518 (Turkey), P.I. 249864 (Greece), P.I. 295608 (Turkey), Beltsville local strain (all ex National Seed Storage Lab., Fort Collins, Colorado, USA), ecotype cultured, Sands Roussillon and var. perpusillon, Finistaire (ex Station Nationale, Versailles, France); *Trifolium affine* L. N.Y.T. 1620 (ex Volcani Centre, Neve Ya'ar, Israel), P.I. 369014 (ex Plant Physiology Institute, Beltsville, USA); *Trifolium repens* var Pitau, var Huia (ex Grassland Division, DSIR, Palmerston North, New Zealand). Plants were either grown in pots in a 50/50 peat–pumice mix with balanced fertilizers or sampled from pure stands in the field. Only fresh tissue was extracted. Both individual and multi-plant samples were extracted.

Extraction of condensed tannins. Plant tissue was frozen in liquid N_2, powdered in a blender or a pestle and mortar, and extracted into 70% aq. Me_2CO [14] containing 0.1% W/V ascorbic acid. Macerate was filtered and the residue re-extracted × 2. Combined filtrates were satd. with NaCl to salt out the Me_2CO (upper phase). The Me_2CO phase was washed with the aq. phase of the extraction solvent which had been satd. with NaCl. The Me_2CO was removed from the washed Me_2CO phase and after addition of an equal vol of H_2O, was extracted × 3 with petrol to remove pigments and lipids. The aq. phase was dialysed against H_2O (3 × 10 l), containing 0.1% w/v ascorbate, through which N_2 (O_2 free) was bubbled. The desalted soln was freeze-dried and stored over Si gel in the dark at 5°.

Purification of condensed tannins. Crude tannins were purified on Sephadex G-50 [8] in Me_2CO–H_2O, (1:1), containing 0.1% w/v ascorbate. Condensed tannins were detected at the void volume of the column by pptn of a procion-blue dye conjugate of bovine serum albumen. Flavanols were detected by the vanillin–HCl test [15]. Me_2CO was removed from the condensed tannin fraction and the aq. soln freeze-dried. Condensed tannin preparation was dissolved in aq. MeOH (1:1) and applied to a small column (3 × 2 cm) of Sephadex LH-20 in aq. MeOH (1:1). Impurities were removed by thorough washing of the column with aq. MeOH (200–500 ml). The condensed tannin was eluted with 50 ml aq. Me_2CO (70%). Me_2CO was removed and the aq. soln freeze-dried. The white voluminous solid was used immediately. Two-D PC in *n*-BuOH–HOAc–H_2O (6:1:2) and 2% aq. HOAc gave only a single spot at the origin when detected with (1) vanillin–HCl (flavanols) (2) NH_3–$AgNO_3$ (3) UV in absence and presence of NH_3, indicating the lack of any mobile organic contaminants.

MW determination. The condensed tannin (10 mg) was dissolved in 0.1 M NaCl containing 0.1% w/v ascorbic acid, dialysed against the same solvent and made up to 5 ml. MW's were determined in a Beckman Model E analytical ultracentrifuge using Rayleigh interference optics as described previously [16,17]. The partial specific vol of condensed tannins in the above solvent at 20° was 0.560 ± 0.005.

Analysis of anthocyanidins. Qualitative. Tannin (40 mg) was mixed with 2 ml *n*-BuOH and 1 ml of conc. HCl in sealed tubes and heated at 100°C for 10 min. The digest was applied as a streak to a sheet of 3 mm paper and developed in Forestal. Anthocyanidin bands were eluted, rechromatographed in Forestal and eluted with MeOH containing 0.01% v/v HCl or EtOH (+0.01% HCl). Anthocyanidins were identified by PC and spectra [18].

Quantitative. Anthocyanidin production was measured for samples which had been boiled in 5% v/v HCl in *n*-BuOH for 2 hr in sealed tubes [19]. Relative amounts of delphinidin to cyanidin were measured, from chromatograms developed in Forestal solvent, by absorption at 545 nm on a Varian Techtron Model 635 spectrophotometer fitted with a scanning attachment. The strips were soaked in glycerol prior to scanning them. A piece of 3 mm filter paper soaked in glycerol was placed in the reference beam. % Anthocyanidin was measured by integrating the area under the peak and expressing it as a percentage of the total area under the delphinidin and cyanidin absorption peaks. *Astringency* of tannins was measured using a modification of a previous method [19]. Tannin soln (1 ml) in 0.1 M Tris–HCl pH 6.5 was added with rapid mixing to 2 ml of diluted (1/500 v/v) ovine blood in 0.1 M Tris–HCl pH 6.5. Samples were chilled in ice for 10 min, centrifuged at 16000 *g* for 10 min, and % protein precipitated was determined.

Acknowledgements—The authors thank Dr. Bate-Smith for helpful discussion, and Dr. J. Lewis for assistance in analysis of the analytical centrifugation plates.

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