# Assessment of the condensed tannin concentration in a collection of *Leucaena* species using $^{14}$C-labelled polyethylene glycol (PEG 4000)

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**Abstract**

Condensed tannin (CT) in 26 *Leucaena* accessions (14 species and 3 hybrids) grown near Townsville, north Queensland was measured using $^{14}$C-labelled polyethylene glycol. Results showed a wide range in the amount of PEG bound (PEG-b) (0–167 mg/g DM) both between and within species, confirming other results indicating that selection and breeding for low CT levels is possible.

Lowest PEG-b values (<25 mg/g) were measured in *L. collinsii, L. lempirana, L. salvadorensis, L. magnifica, L. trichodes* and *L. trichandra* OFI 4/91. Highest values (>120 mg/g) were measured in *L. pulverulenta, L. involucrata, L. diversifolia* K156 and OFI 82/92, and *L. trichandra* CPI 46568. *L. pallida, L. diversifolia* CPI 33820, *L. trichandra* OFI 53/88, *L. macrophylla istmensis*, the KX2 F1 hybrid between *L. pallida* and *L. leucocephala* and *L. leucocephala* had intermediate values. The cultivars Cunningham and Tarramba and the *L. pallida* × *L. leucocephala* F1 hybrid had similar values (81–87 mg/kg).

The results were compared with reported values from the same species grown in south-east Queensland and in Honduras where the CT was measured by different techniques (Dalzell *et al*. 1998; Stewart and Dunsdon 1998). For the Honduran data, with 12 common accessions, there was an excellent linear relationship between PEG-b and total tannin estimated by radial diffusion protein-precipitation assay ($r^2$ = 0.881), but a poorer relationship with CT estimated by a butanol/HCl assay ($r^2$ = 0.649). For the south-east Queensland data, the linear relationship between CT estimated by a butanol/HCl method and PEG-b was significant ($r^2$ = 0.60), but there were notable departures from the relationship. Reasons for these are discussed.

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## Introduction

Condensed tannins (CT) are present in many fodders. At low concentration (1%), they may be beneficial in reducing bloat and providing some bypass protein to the small intestine. At higher concentrations, their presence may result in lower acceptance by animals, reduced digestibility of plant proteins, adverse effects on rumen bacteria and gut enzymes, and damage to the gut wall with consequent impairment of nutrient uptake (Silanikove *et al*. 1994; Kumar and D'Mello 1995; Barry and McNabb 2000; Norton 2000). Tropical leguminous shrub and tree legumes can have high levels of CT (Norton 2000), so it is advisable to screen for these in any evaluation program designed to release new cultivars to the grazing industry.

Screening for CT in plants is complicated for several reasons. The heterogeneous nature of CTs and their instability, together with the lack of any satisfactory standard, make it difficult to assess them chemically (Hagerman and Butler 1989). Furthermore, the concentration determined by any particular method may not reflect the activity of the CT in modifying nutritive value. The different reactivities from various species, and possibly cultivars, mean that there is no universal reference material. Using external reference standards or an inappropriate technique can, therefore, lead to serious bias in assessing CT concentration (Jones *et al*. 2000; Jones and Palmer 2000). Obtaining internal standards is time-consuming and costly and may require relatively large quantities of plant material. Using internal standards is therefore not appropriate for screening different genera and species. The alternative is to use a common standard; for example, in New Zealand CT from *Lotus pedunculatus* was used to screen a large number of tropical legumes (Jackson *et al*. 1996), and Dalzell *et al*. (1998) have used the CT from *Leucaena pallida* to screen a range of *Leucaena* species.

A recent approach to assessing CT in plant material is based on the ability of polyethylene glycol (PEG) to bind to CT to form insoluble complexes. Using $^{14}$C-labelled PEG 4000 it is possible to assess the amount of PEG bound to the tannin when a solution of PEG is mixed with plant material (Silanikove *et al.* 1996). This assessment has given excellent results with a range of tropical shrub legumes (Jones and Palmer 2000). We used this method to assess the variation in CT in a collection of *Leucaena* species.

## Materials and methods

Twenty-six accessions from 14 *Leucaena* species (Table 1) were grown in rows 3 m apart with plants spaced at 50 cm on a Kandisol (red earth) at the CSIRO Research Station, Lansdown, north Queensland. Details of the experimental procedures and the results have been published (Jones 1998).

At the end of the 4-yr cutting experiment, 6-week regrowth was sampled in June 1999 taking the terminal portion of actively growing shoots, including 3 fully expanded leaves and the growing tip. The samples were immediately dried at 65$^{o}$ C in a dehydrator for 48 h and then ground to pass a 1 mm screen. The following day, duplicate samples were measured for PEG-binding capacity, using a modification of the method of Silanikove *et al*. (1996). We used 0.5 g plant material in 15 ml of labelled PEG solution [32 g PEG 4000 in 1 L 0.05 M Tris-BASE buffer (Sigma) at pH 7.1, spiked with 50 μCi of $^{14}$C-labelled PEG 4000 plus 2 L distilled water]. This is half of the sample weight used in the published method as we had difficulty getting a clean supernatant with the recommended method of 1.0 g in 15 ml solution. The rate of PEG 4000 is also lower than the published level since we had shown that ≈ 320 mg PEG/g sample was adequate for the materials we were using (Palmer and Jones 2000). Tubes were stoppered and placed in an end-over-end shaker for 24 h, then centrifuged at 2500 G for 30 min. Two samples, each of 1 ml, were withdrawn from the supernatant and added to 10 ml of scintillant (Opti-Phase "HiSafe" 3 ®; Fisher Chemicals, England). Samples were subsequently counted in a β Scintillation counter (Wallac 1410, Pharmacia, Finland) for 10 min ($C_{sm}$). The radioactivity of 1 ml of the PEG solution in 10 ml of scintillant served as the standard ($C_{st}$) and that of 1 ml of the Tris buffer in 10 ml scintillant as the blank ($C_{bl}$). The weight of PEG in the tubes ($A_{PEG}$) was 160 mg. The amount of PEG 4000 bound to the sample (PEG-b) was calculated from the counts (C) by the formula:

$$\text{PEG-b} = (C_{st} - C_{bl}) - (C_{sm} - C_{bl}) \text{ x } A_{PEG} / [(C_{st} - C_{bl}) \times \text{sample weight}]$$

The results were expressed as mg PEG/g DM. Regression analysis was used to compare the results from 24 accessions with the total and extractable CT results for these same accessions grown at Redland Bay in south-east Queensland obtained by a modified butanol/HCl method (Dalzell and Kirven 1998). Results for 12 accessions were also compared with those for the same accessions grown in Honduras that were analysed by a butanol/HCl assay for CT and by a radial diffusion protein-precipitation technique (Hagerman 1987) using bovine haemoglobin, for total tannin (Stewart and Dunsdon 1998).

## Results

There was a wide range in PEG-b values (0–167 mg/g DM) across the accessions (Table 1). Several had values close to zero, namely: *L. trichandra* OFI 4/91, *L. lempirana* OFI 5/91, *L. collinsii collinsii* OFI 52/88, *L. collinsii zacapana* OFI 56/88, *L. salvadorensis* OFI 36/88 and *L. magnifica* OFI 19/84. At the other extreme, *L. pulverulenta* OFI 83/87, *L. diversifolia* OFI 82/92 and K156, *L. trichandra* CPI 46568 and *L. involucrata* OFI 87/92 had high PEG-b values. The two commercial cultivars of *L. leucocephala*, Cunningham and Tarramba, had intermediate values that were similar to those of *L. pallida* OFI 79/92, the CSIRO composite, the KX2 F1hybrid and *L. trichandra* OFI 53/88.

The linear relationship between the extractable CT (y; mg/g) as measured by the butanol/HCl method and PEG-b (x; mg/g) was: $y = 0.935x - 11.20$; $r^2 = 0.602$; $P < 0.0001$ (Figure 1). At low

**Table 1.** Mean PEG-binding (PEG-b) values (mg/g DM) for 26 *Leucaena* accessions and the condensed tannin (CT) estimates (butanol/HCl) for 24 of these accessions (Dalzell *et al*. 1998).

| Accession | Species | Identity | CT estimates | | |
|---|---|---|---|---|---|
| | | | PEG-b | UQ ECT[1] | UQ TCT[2] |
| | | | (mg/g) | (mg/g) | (mg/g) |
| 1 | *L. collinsii collinsii* | OFI[3] 52/88 | 7 | 0 | 1 |
| 2 | *L. collinsii zacapana* | OFI 56/88 | 11 | 0 | 2 |
| 3 | *L. diversifolia* | OFI 82/92 | 153 | 62 | 100 |
| 4 | *L. diversifolia* | OFI 83/92 | 100 | 115 | 126 |
| 5 | *L. trichandra* | OFI 4/91 | 0 | 2 | 4 |
| 6 | *L. trichandra* | OFI 53/88 | 86 | 158 | 181 |
| 7 | *L. esculenta* | OFI 47/87 | 104 | 100 | 112 |
| 8 | *L. pallida* | OFI 52/87 | 53 | 18 | 24 |
| 9 | *L. pallida* | OFI 79/92 | 83 | 42 | 53 |
| 10 | *L. involucrata* | OFI 87/92 | 135 | 135 | 148 |
| 11 | *L. lanceolata lanceolata* | OFI 43/85 | 40 | 8 | 13 |
| 12 | *L. lempirana* | OFI 5/91 | 4 | 2 | 3 |
| 13 | *L. macrophylla istmensis* | OFI 47/85 | 57 | 6 | 12 |
| 14 | *L. pulverulenta* | OFI 83/87 | 167 | 137 | 154 |
| 15 | *L. salvadorensis* | OFI 36/88 | 12 | 0 | 1 |
| 16 | *L. magnifica* | OFI 19/84 | 13 | 0 | 1 |
| 17 | *L. trichodes* | OFI 61/88 | 21 | 1 | 3 |
| 18 | *L. leucocephala* | cv. Tarramba | 81 | 12 | 19 |
| 19 | *L. leucocephala* | cv. Cunningham | 87 | 5 | 9 |
| 22 | *L. pallida* × *L. leucocephala* | KX2 (F1, K748 X K636)[4] | 84 | 41 | 53 |
| 23 | *L. diversifolia* | K156 | 144 | 151 | 167 |
| 24 | *L. pallida* | CSIRO composite | 89 | 63 | 79 |
| 25 | *L. diversifolia* | CPI 33820[5] | 68 | 118 | 137 |
| 26 | *L. trichandra* | CPI 46568 | 121 | 164 | 189 |
| 20 | *L. pallida* × *L. leucocephala* | KX2 (F5, K376 X K8) | 116 | | |
| 21 | *L. diversifolia* × *L. l* | KX3 (F2, K156 X K8) | 115 | | |

[1]Extractable CT.
[2]Total CT from Dalzell *et al*. (1998).
[3]Oxford Forestry Institute code.
[4]K numbers are codings of the University of Hawaii.
[5]CPI Commonwealth Plant Introduction number.

and intermediate levels of CT there was a tendency for levels of CT measured by the butanol/HCl method to underestimate and at high levels of CT to overestimate compared with levels measured by PEG-b (Figure 1). The relationship between total CT (y; mg/g) and PEG-b (x; mg/g) was similar to that for extractable CT: $y = 1.08x - 11.1$; $r^2 = 0.645$; $P < 0.0001$. This was expected in the light of the very close linear relationship between total (y) and extractable (x) CT: $y = 1.11x + 4.25$; $r^2 = 0.991$; $P<0.001$).

There were some marked departures from the overall linear relationship. In particular, *L. macrophylla istmensis* (13), the 2 *L. leucocephala* cultivars, Tarramba (18) and Cunningham (19), and *L. diversifolia* OFI 82/92 (3) had very low values with the butanol/HCl method. Three accessions (6, 25, 26) comprising 2 *L. trichandra* and 1 *L. diversifolia* had very high levels with the butanol/HCl method. Those accessions with little or no CT were detected by both methods.

For the 12 accessions that were common to the Honduran study, total tannins (y) and CT ($y^1$) were linearly correlated with PEG-b (x):

$y = 6.63 + 2.42x$ ($r^2 = 0.881$; $P<0.0001$), and

$y^1 = 24.7 + 1.37x$ ($r^2 = 0.649$; $P = 0.0016$) (Figure 2).

## Discussion

The range of PEG-b values (0–167 mg/kg DM), reflecting a wide range of CT both between and within species, shows how variable this character is within the *Leucaena* genus. Such a wide range has been reported earlier (Dalzell *et al*. 1998; Stewart and Dunsdon 1998) and our results are generally in line with theirs. However, the relationship with the south-east Queensland data of Dalzell *et al*. (1998) is not strong. This may reflect differences between the studies. Firstly, the sample tissue was different for the 2 sites. In

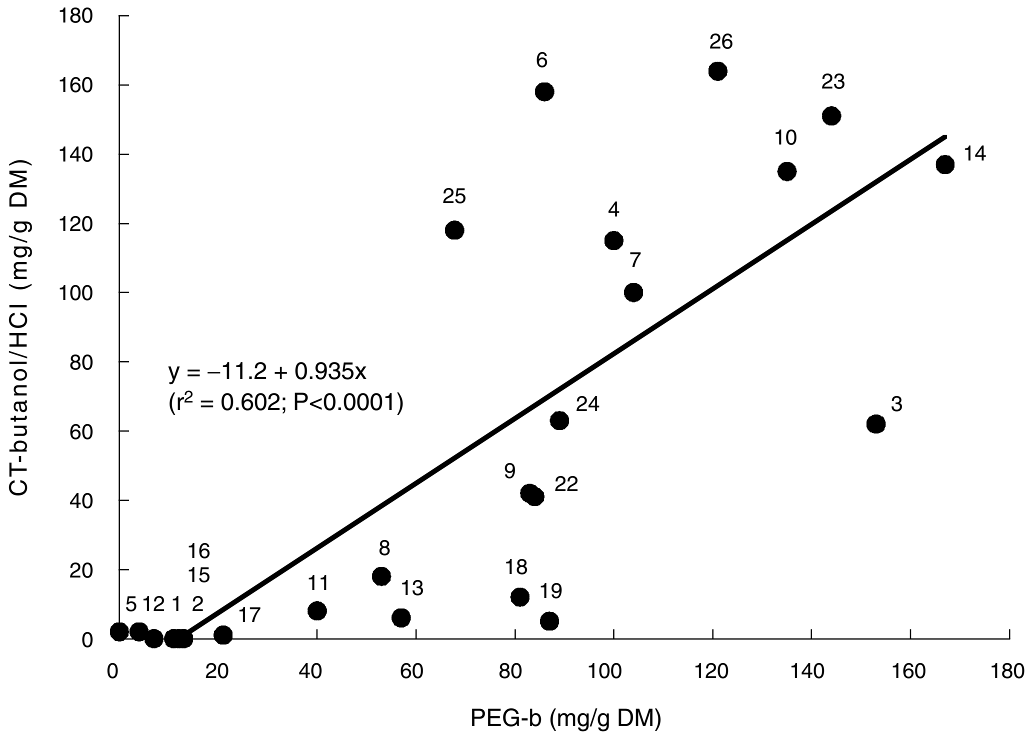


**Figure 1.** The relationship between condensed tannin (CT) measured on 24 *Leucaena* accessions in south-east Queensland by a butanol/HCl method and the PEG-binding (PEG-b) values for the same accessions grown in north Queensland. The numbers refer to the accessions listed in Table 1.

the south-east Queensland study, the youngest fully expanded leaf (YFEL) on actively growing shoots was chosen compared with the terminal portion of the shoot down to and including the third fully expanded leaf in our study. Young leaves are known to have higher CT than older leaves and older stems. Secondly, they used freeze-dried samples and we used oven-dried samples. Since drying treatment appears to have little effect on the PEG-b values (Silanikove *et al.* 1996), it is unlikely that method of drying was responsible for the differences noted. Thirdly, samples were taken at different times of the year for the 2 sites, and it is known that growing conditions can affect the levels of CT in plant tissues. Although the actual values for the estimates may differ, there is usually a similar ranking between accessions sampled at different times of the year (Dalzell 2000) and a closer relationship could have been expected. Finally, the standard CT used as a reference can greatly influence the estimates by introducing bias. In the study of Dalzell *et al.* (1998), the standard CT was from *L. pallida* whereas, with the PEG-b technique, no standard is necessary since the active sites on any CT appear to be preferentially bound by the PEG. If PEG binds to fractions other than CT, this method would overestimate CT levels. This appears not to be an issue since *in vitro* DM and N digestibilities on CT-free forage in the presence or absence of PEG were similar (Jones and Palmer 2000).

In the Honduran work, no standards were used due to the uncertainty of choice of a suitable standard. Their results were expressed as optical density (butanol/HCl assay) and ring area (radial diffusion assay). However, the excellent linear relationship between the protein-precipitation radial diffusion assay and the PEG-b assay, despite the differences in sites and sample preparation, suggests that the relative ranking of accessions across sites for tannin levels is fairly consistent and associated more with genetics than with site characteristics.

It is important that estimates of CT should have biological relevance to feed quality. However, few studies have related these parameters. *In vitro* N digestibility (IVND) for a range of

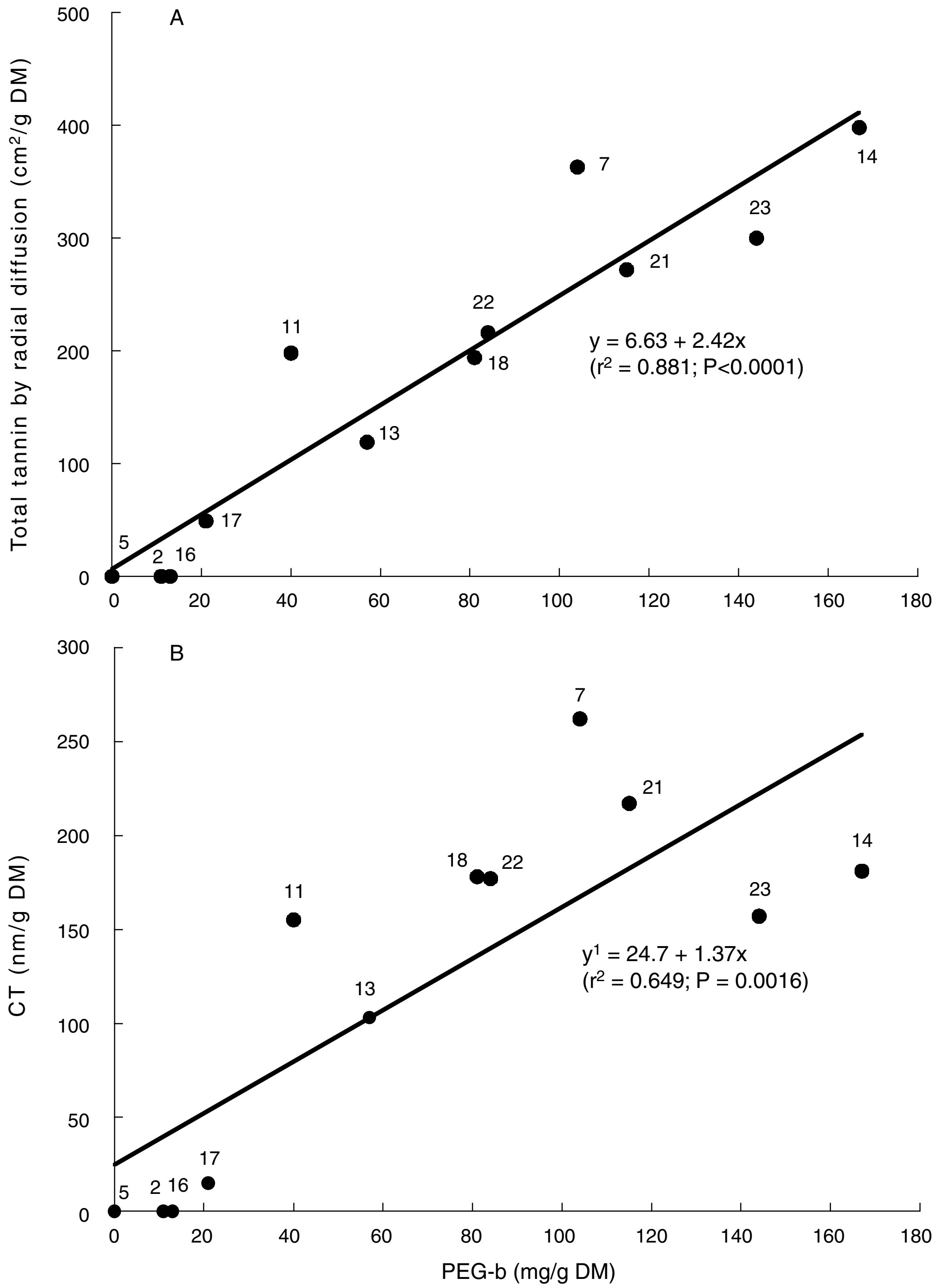


**Figure 2.** The relationship between: A. total tannin measured by a protein-precipitation radial diffusion assay on 12 *Leucaena* accessions grown in Honduras and the PEG-binding (PEG-b) values for the same accessions grown in north Queensland; and B. condensed tannin (CT) measured by a butanol/HCl assay on 12 *Leucaena* accessions grown in Honduras and the PEG-binding (PEG-b) values for the same accessions grown in north Queensland. The numbers refer to the accessions listed in Table 1.

tropical shrub legumes varying in CT concentration was negatively related ($r^2 = 0.973$) to PEG-b (Jones *et al.* 2001) indicating that the PEG-b values have biological significance. Furthermore, *in vivo* N digestibility was also negatively and linearly related to level of CT in *Leucaena* diets fed to sheep (McNeill *et al*. 2000). Relationships of IVND with CT measured with vanillin/HCl or butanol/HCl calculated from the data of Jones *et al*. (2001) were not so well correlated: $r^2 = 0.674$ and 0.014, respectively. The legumes in that study were from 3 genera, *Leucaena*, *Calliandra* and *Acacia.* When only the 3 *Leucaena* species were used, the relationship between IVND and CT measured by the butanol/HCl method was greatly improved from $r^2 = 0.0007$ to $r^2 = 0.875$ (Jones *et al*. 2001). We consider that the poorer correlations were due to the use of a common standard for all genera.

Current methods for *in vitro* analysis of tannins are inadequate for measuring the amounts of tannins present or their activity (Waterman and Mole 1994; Schofield *et al.* 2001), and different methods of analysis have given variable results. It is not known what bias may be introduced if CT from one species within a genus is used as a standard for comparing a range of other species in the same genus. However, data from our study give some indication. Within the 3 *L. pallida* accessions, the correlation between CT levels by butanol/HCl and PEG-b was higher ($r^2 = 0.896$) than that for all *Leucaena* accessions ($r^2 = 0.602$).

The CT levels for the cultivars Cunningham and Tarramba by the modified butanol/HCl method (Dalzell *et al*. 1998) are much lower than other published results for *L. leucocephala* (Wheeler *et al*. 1995; Jackson *et al*. 1996; McNeill *et al.* 1998; Balogun *et al*. 1998). This could be due to the difference in CT characteristics between *L. leucocephala* and *L. pallida*. Relative to the CT from *L. leucocephala,* the CT from *L. pallida* produces more anthocyanidin /mg (Dalzell and Kirven 1998); hence, if the CT from *L. pallida* is used as the standard, CT will be underestimated compared with values obtained if CT from *L. leucocephala* was used. This may explain the low estimates for CT in the cultivars of *L. leucocephala* measured by Dalzell *et al*. (1998).

Compared with earlier work (Jones and Palmer 2000), the PEG-b values for both *L. leucocephala* cv. Cunningham and *L. trichandra* CPI 46568 were higher (≈64%) in the present study (87 *vs* 53 and 121 *vs* 74, respectively). This higher level of CT may be explained by the younger age of leaves sampled (terminal 3 leaves and shoot apex compared with the terminal 5 leaves), and the cooler temperatures when samples were taken in our study (Dalzell *et al.* 1998). Furthermore, the PEG-b levels in *L. pallida* were closer to those of *L. leucocephala*, but a different accession of *L. pallida* was used in the earlier work.

It is not known why the PEG-b values appear to give such a good estimate of 'tannin activity' across different species and genera and the biochemical reasons for this need to be further explored. The method certainly overcomes a major problem in the most common butanol/HCl assay of choosing a suitable reference CT as well as a number of other factors associated with colour development (Schofield *et al*. 2001; Silanikove *et al*. 2001).

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