The effect of sample drying conditions on estimates of condensed tannin and fibre content, dry matter digestibility, nitrogen digestibility and PEG binding of *Calliandra calothyrsus*

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**Abstract**

Leaf samples of the browse shrub *Calliandra calothyrsus* were used to study the effect of temperature of drying (25, 45, 65, 85 and 105°C), and method of drying, either in dry air (aerobic) or in dry nitrogen (anaerobic), on estimates of fibre, condensed tannin (CT), digestibility and PEG binding. These estimates were also made on freeze-dried samples. Correlations were made between these variables. In general, there was much less change with increasing temperature for samples dried anaerobically than for samples dried aerobically for all measures. For the condensed tannin measures (using the butanol–HCl with tannins isolated from *C. calothyrsus* as a standard), there was an interaction of method \(\times\) temperature of drying \(p < 0.01\) to \(p < 0.0001\). At higher temperatures there was more bound tannin (protein- and fibre-bound) with aerobic drying. Free tannin under anaerobic conditions increased slightly with increasing temperature whereas there was a large linear decrease under aerobic drying. A similar response was obtained by the protein precipitation method though the mean level of free tannin using this method was much lower than by the butanol–HCl method (11% vs 21%). Total CT (TCT) increased slightly with temperature under anaerobic drying, but decreased by a similar magnitude (about 10%) with aerobic drying. The freeze-dried samples had values similar to samples dried at the lower temperatures (25 and 45°C) for all measures. The mean TCT for *C. calothyrsus* in this study of 29% is far higher than those reported in other studies using unrelated tannin standards.

Acid detergent fibre (ADF) was higher for the aerobically dried samples, and levels for both drying methods increased slightly with temperature. For neutral detergent fibre (NDF), levels rose at temperatures above 45°C; the rise with aerobic drying was much greater and gave a significant interaction \(p < 0.01\). Both the N% in NDF and the total N in NDF were higher in the aerobically drying conditions.

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dried samples. For both measures, levels declined as temperature increased from 25 to 45°C and then increased. Levels for the freeze-dried samples were similar to those anaerobically dried at 25 and 45°C. In vitro dry matter and N digestibility (IVDMD and IVND) were measured by a modified Tilley and Terry [J. Br. Grassld. Soc. 18 (1963) 104] technique using ±PEG with 14C-labelled PEG to correct IVDMD for PEG-tannin complexes in the residue (CIVDMD). With anaerobically dried samples there was no change with temperature for IVDMD, CIVDMD, or IVND in the presence of PEG (IVNDP). However, under aerobic conditions, for most measures of digestibility, levels declined above 45°C. PEG binding was unaffected by temperature of drying but, was lower \( p < 0.001 \)† for aerobic drying compared with anaerobic drying, and far higher for freeze-dried than for oven-dried samples. These data support the hypothesis that the degree of complexing of CT with components of the plant is affected by both temperature and oxidation; the latter having the greater effect particularly at high drying temperatures. Where facilities for freeze drying are not available, drying at 45°C would appear to be the best option. © 2000 Elsevier Science B.V. All rights reserved.

**Keywords:** *Calliandra calothyrsus*; Condensed tannin; PEG binding; Digestibility; Fibre analysis

1. **Introduction**

Drying is known to modify the assessment of tannins and the assessment of fibre fractions in tanniferous plants (Makkar and Singh, 1995; Lowry et al., 1995). Furthermore, the nutritive value of some tanniferous fodders can also be greatly influenced by drying method (Mahyuddin et al., 1988; Ahn et al., 1989; Palmer and Schlink, 1992). Most comparisons have been made between freeze drying (essentially anaerobic) and oven drying (aerobic). Some of the effects with oven drying are due to oxidative changes and these could be obviated if samples are dried in an oxygen-free environment.

The objective of this study was to measure the effects of drying, at a wider range of temperatures than studied previously, in both aerobic and anaerobic conditions on measures of fibre fractions, CT (condensed tannin) levels and digestibility in the presence and absence of PEG 4000. The effects of these treatments on PEG binding (Silanikove et al., 1996) were also measured.

2. **Materials and methods**

2.1. **Plant samples**

Leaf samples were obtained in November from well established *Calliandra calothyrsus* (CPI 115690) shrubs, growing under irrigation, in rows 3 m apart at the CSIRO Lansdown Research Station, 50 km south of Townsville, North Queensland, Australia (19°40' S, 146°50'E). Samples consisting of the terminal 5 fully expanded leaves of actively growing shoots were transported from the field over liquid nitrogen in an insulated container. Samples were either freeze-dried or dried in an oven at temperatures
of 25, 45, 65, 85 and 105°C in a flow of dry compressed air (aerobic) or dry compressed nitrogen (anaerobic), the gas flow being 2.0 l/min. The 25°C sample was dried for 72 h and the higher temperature samples for 48 h. After drying, all samples were ground to pass a 1 mm screen, equilibrated under vacuum at 25°C for 48 h to bring them to the same moisture content, then stored prior to analysis in sealed containers at −72°C. All samples had moisture contents of less than 0.25%.

2.2. Condensed tannin analyses

Free (FCT), protein-bound (PBCT), fibre-bound (FBCT) and total CT (TCT) were extracted by the method of Terrill et al. (1992) and measured by the butanol–HCl method described by Jackson et al. (1996). FCT was also determined by the protein precipitation technique (FCTpp) using bovine serum albumin as described by Hagerman and Butler (1978). The technique was modified by using 70% acetone instead of methanol. After extraction, acetone was evaporated and the residue extracted with ether, leaving the tannin in the aqueous layer. The CT used as a standard for both techniques was extracted from *C. calothyrsus* (CPI 115690) and purified on Sephadex LH-20. The purified CT was freeze-dried and stored in a desiccator at −20°C until required. This CT has minimal contamination with hydrolysable tannin (HT) since *C. calothyrsus* has very low levels (<0.3%) of HT (Wina et al., 2000).

2.3. Fibre analyses

Acid detergent fibre (ADF) and neutral detergent fibre (NDF) were determined using the methods described by Van Soest et al. (1991). Nitrogen in the NDF (N%_NDF) was measured using the Kjeldahl method (AOAC, 1980); total N in the NDF (N_NDF) was calculated by multiplying N%_NDF by the weight of NDF residue.

2.4. Digestibility

In vitro digestion studies were conducted on the samples using a modified Tilley and Terry (1963) two-stage digestion method in which Stage 1 was 72 h and Stage 2 of 24 h duration. Plant samples were digested with and without PEG 4000. The use of labelled PEG 4000 enabled the amount of PEG (as complexes) in the residue to be estimated and used to calculate a corrected dry matter digestibility (CIVDMD). Measurement of the N in both plant sample and in the residues after Stage 2 digestion also enabled N digestibility to be calculated (IVND) (Palmer and Jones, 2000).

2.5. PEG binding

Duplicate plant samples were measured for PEG binding (PEG_BS) following the method of Silanikove et al. (1996). However, we used 15 ml, instead of 7.5 ml, of a solution of PEG 4000 in tris–buffer (pH 7.1) spiked with labelled PEG 4000, and used an end-over-end shaker running continuously for 24 h. The amount of PEG in the tubes was, however, the same as for the published method. PEG binding was also measured on the
residues following Stage 1 of the Tilley and Terry (1963) method (PEG_BR) by subtracting the estimated PEG in the supernatant from the total PEG added.

2.6. Statistical analyses

Results from duplicate samples taken in the field were analysed using ANOVA. With the tannin estimates, field replicates and laboratory replication (2×) were included. Correlation coefficients between variables were calculated using all treatments with two replicates (22 d.f.).

3. Results

3.1. Condensed tannin content

For FCT measured by butanol–HCl (Fig. 1a), there was a significant interaction \( p < 0.001 \) between aerobic/anaerobic condition and temperature of drying. Levels increased with drying temperature from 21.8 to 25.0% \( (\text{LSD } 5\% = 2.4\%) \) for anaerobic, but there was a marked decrease from 23.8 to 14.6% for the aerobic condition. There were no differences in FCT for samples dried at 25°C and freeze-dried material. There

Fig. 1. Effect of drying condition on: (a) FCT and FCTpp; (b) PBCT; (c) FBCT; (d) TCT in *C. calothyrsus* leaf. (■) Aerobic; (○, ○) anaerobic; (▲, △) freeze-dried sample.
was a similar trend for FCTpp, however, the mean level was markedly lower than that for FCT (11% vs 21%).

For PBCT (Fig. 1b), there was an overall increase with temperature of drying for the aerobic condition from 3.3 to 5.0% (LSD 5% = 0.82) and a decrease (4.5 to 2.8%) for anaerobic drying. The interaction was significant ($p < 0.001$). The freeze-dried sample had a PBCT of 3.9% and was only different from that measured at 105°C dried under anaerobic conditions. FBCT (Fig. 1c), increased more with aerobic drying than with anaerobic drying, the interaction being significant ($p < 0.001$). Under aerobic conditions the FBCT increased from 2.8 to 6.6% (LSD 5% = 0.5), whereas, under anaerobic conditions it increased from 2.1 to 4.1%, the major increases being above 65°C. The freeze-dried sample had an FBCT of 2.0% which was lower than for all samples dried aerobically but was only different to those dried anaerobically above 65°C ($p < 0.05$). Under anaerobic conditions the total bound CT (PBCT + FBCT) did not change with increasing drying temperature (mean = 6.7%). Whereas, under aerobic conditions it increased from 6.0 to 11.6% (LSD 5% = 0.69). The freeze-dried sample had a total bound CT of 5.9%. The interaction was significant ($p < 0.001$).

With the three fractions combined to give total CT (TCT), the major effect was on the aerobic/anaerobic condition with a significant interaction with temperature ($p < 0.01$). With aerobic drying, TCT declined with increasing temperature from 29.4 to 26.2% (LSD 5% = 2.4%), whereas with anaerobic drying, TCT increased from 28.4 to 31.8%. The freeze-dried sample had a TCT of 29.8% which was not significantly different from anaerobic samples dried at all temperatures but was higher than the aerobically dried samples at 65 and 105°C (Fig. 1d).

3.2. Fibre fractions

The effects of drying condition and temperature on ADF were small. There was a significant ($p < 0.001$) effect of increasing drying temperature (19.9–23.3%) (LSD 5% = 1.6%), and a significant ($p < 0.001$) difference between anaerobic and aerobic drying (22.8–20.5%) (LSD 5% = 1.0%). The interaction was not significant ($p > 0.05$). The freeze-dried sample had an ADF of 19.1% (Fig. 2a).

For NDF there was a significant ($p < 0.05$) interaction between aerobic/anaerobic condition and temperature of drying. Under aerobic conditions the NDF increased from 33.5 to 43.8% (LSD 5% = 3.5), whereas under anaerobic conditions the increase was lower with a significant increase only at the highest temperatures (33.7–36.8%). At the higher temperatures NDF was markedly higher for the aerobic drying. The freeze-dried sample had an NDF content of 32.1%. This was not significantly different to anaerobically dried samples below 105°C or to aerobically dried samples at 25 and 45°C (Fig. 2b).

The N%_NDF was significantly higher for the material dried under aerobic conditions than under anaerobic conditions (3.8% vs 2.7%) (LSD 5% = 0.5%). For both aerobic and anaerobic the effect of increased drying temperature was an initial decrease in nitrogen from 3.3% at 25°C to 2.7 and 3.0% at 45 and 65°C, followed by an increase to 3.6 and 3.7% at 85 and 105°C. There was no interaction. The freeze-dried sample had an N%_NDF content of 2.8% (Fig. 2c).
The N\_NDF was significantly higher \((p < 0.001)\) with aerobic drying than for anaerobic drying (14.8 mg/g vs 9.5 mg/g) \((\text{LSD 5\%} = 1.3 \text{ mg/g})\). There was an overall reduction with increasing drying temperature from 25 to 45°C (11.2–8.8 mg/g) \((\text{LSD 5\%} = 2.0 \text{ mg/g})\), and then an increase to 15.3 mg/g at 105°C. The freeze-dried sample had an N\_NDF of 9.0 mg/g. The interaction between temperature and aerobic/anaerobic condition was not significant \((p > 0.05)\).

3.3. Digestibility

There was an interaction between method of drying and temperature for IVDMD \((p < 0.05)\). For anaerobic drying, IVDMD did not change with increasing temperature of drying \((\text{mean} = 55.3\%, \text{LSD 5\%} = 4.8\%)\). However, for aerobic drying, IVDMD decreased from 56.8% at 25 and 45°C to 49.6% at the higher temperatures. The freeze-dried sample had an unexpectedly low value of 47.2%.

There was also an interaction between method of drying and temperature for CIVDMD \((p < 0.01)\). For aerobic drying, as temperature of drying increased from 25 to 85°C, CIVDMD fell from 63.7 to 55.5% then rose to 60.3% at 105°C \((\text{LSDs 5\%} = 2.2\%)\). For anaerobic drying, there was no change in CIVDMD with temperature; the mean value was 64.7%. Freeze-dried material had a CIVDMD content of 63.5% \((\text{Fig. 3b})\). For IVND, without PEG addition, there were no significant
differences ($p > 0.05$) for either aerobic or anaerobic condition or temperature of drying (overall mean = 54.4%).

For N digestibility in the presence of PEG (IVNDP), however, there was a significant interaction ($p < 0.05$) between aerobic/anaerobic condition and temperature of drying (LSD 5% = 3.4%). Under anaerobic conditions, IVNDP decreased from 84.9 to 82.1% with increasing temperature of drying, whereas, under aerobic conditions IVNDP decreased more rapidly from 84.2 to 77.3%. For the freeze-dried sample IVND was 88.0% (Fig. 3c).

The improvement in N digestibility due to PEG addition (IMPIVND) did not change with temperature of drying ($p > 0.05$), but was significantly higher ($p < 0.01$) under anaerobic conditions (32.2% vs 23.2%) (LSD 5% = 4.3%). Improvement for the freeze-dried sample was greater than for either method of oven drying owing to the low value for IVND measured in the absence of PEG (Fig. 3d).

3.4. PEG binding

The amount of PEG bound to the residue (PEG_BR) was higher for samples dried anaerobically than for those dried aerobically and the interaction between aerobic/anaerobic condition and temperature of drying was highly significant ($p < 0.001$). This
interaction was mainly due to a marked drop (24%) in PEG binding with increased temperature of drying from 25 to 45°C for the aerobic condition (Fig. 4a). Freeze drying gave the highest PEG_BR value of 106.8 mg/g. With PEG_BS there was no effect of temperature of drying but the anaerobic condition gave higher values than the aerobic (111.3 vs 95.0, \( p < 0.001 \)) (Fig. 4b). The PEG_BS value for the freeze-dried sample was 137 mg/g.

4. Discussion

4.1. Condensed tannin content

There are several mechanisms by which CT can react with macromolecules in the plant material (Makkar and Singh, 1995; Makkar et al., 1995a,b; Lowry et al., 1995). These include the denaturing of plant enzymes (e.g., proteases), oxidative processes mediated through quinones, leading to irreversible covalent links with, e.g., protein, or heat treatment per se favouring chemical bond formation between tannins and other macromolecules. The act of drying may bring the CT in close proximity to potentially recipient macromolecules and the reaction may then only proceed when the material is rehydrated.

The only difference between freeze-dried samples and samples dried at 25°C either aerobically or anaerobically was for PBCT measured on samples dried anaerobically. This suggests that any enzymic effects were minimal as it is expected that heating at 25°C for 72 h would be most likely to have a major effect.

The trends for FCT and FCTpp with changing temperature and aerobic/anaerobic condition were remarkably similar (\( r = 0.873^{***} \)). They were, however, significantly lower for FCTpp than for FCT (11% vs 22%). This may mean that the chromatographically purified standard is not truly representative of the CT extract from the plant and that may favour a fraction that is less reactive to protein precipitation or has

Fig. 4. Effect of drying condition on: (a) PEG_BR and (b) PEG_BS by \( C. \) calothyrsus leaf. (■) Aerobic; (○) anaerobic; (▲) freeze-dried sample. (A) Main effect of atmosphere and (T) drying temperature with levels of significance.
an anthocyanin composition giving a different colour development profile than that in the plant extract. The protein chosen will also affect the magnitude of any difference between the two techniques.

The major change in both FCT and FCTpp was with aerobic drying, suggesting the strong influence of oxidative processes in binding free tannins. Under anaerobic conditions, any changes in FCT, although significant, were minor. This is supported by changes in PBCT with increasing drying temperature that showed an increase for aerobic drying (1.7%) and a decrease for anaerobic drying (1.7%). This decrease in PBCT with anaerobic drying was offset by a linear increase in FBCT (2.0%). Under aerobic conditions, the FBCT increased with drying temperature by 3.8%, the majority of this increase being above 65°C. This finding is at variance with the results of Makkar and Singh (1995), using Mediterranean browses, who found a decrease in CT content associated with both ADF and NDF at 100°C compared with the values for freeze-dried and samples dried at 50°C. The total bound tannin (PBCT + FBCT) was not affected by temperature of drying under anaerobic conditions, whereas the marked increase under aerobic conditions again indicates the dominant effect of the oxidative processes.

The net result of these changes was an increase in TCT of 3.4% with increased drying temperature under anaerobic conditions that was similar to the measured increase in FCT (3.2%). Whereas, under aerobic conditions TCT fell by 3.2% owing to a drop of 8.7% in FCT, there was an increase of 1.7% in PBCT and an increase of 3.8% in FBCT. These data indicate that with aerobic drying there was a major effect of the oxidative processes leading to a portion of tannin being irreversibly bound and so not extracted by the analytical procedure. Further work is required to evaluate any changes in the anthocyanin make-up of the various fractions identified in this study. These data show that low temperatures of drying under anaerobic conditions are desirable when evaluating the CT fractions of plant material. The use of freeze-dried material, where the oxygen is excluded immediately after harvest, is the preferred and most practical option.

It is interesting to note that the levels of the CT fractions measured in this study are far higher (approximately fivefold) than those reported for similar plant material in earlier work with C. calothyrsus where tannin from Lotus pedunculatus was used as a standard (Jackson et al., 1996). This difference can be explained by our use of an appropriate standard, extracted from the same plant material. It again emphasises the importance of using suitable standards, particularly in screening procedures for potential forage plants.

4.2. Fibre fractions

The minor increase in ADF and NDF under anaerobic conditions with increasing temperature of drying was associated with no increase in bound CT in these samples. However, under aerobic conditions there was a marked increase in NDF (10% units) and a smaller increase in ADF (4% units). The NDF therefore increased by about 30% and the ADF by about 20%, reflecting the increase in CT bound to protein and possibly other cell wall constituents in the fibre fractions as found in other studies (Makkar et al., 1995a,b). The higher levels of N%_NDF and N_NDF with aerobic drying support the hypothesis that these are due to oxidative processes, the nitrogen content under aerobic conditions being 50% higher than that under anaerobic drying.
Again the data suggest that air drying is not an appropriate method of drying tanniniferous plant material for analyses and support the findings of Makkar and Singh (1995). If, however, freeze drying is not an option, then drying below 65°C would be an alternative although measures of nitrogen would be in error.

4.3. Digestibility

The major effect of temperature of drying appears to be associated with oxidative changes since there was virtually no response with samples dried anaerobically. The lower digestibility with aerobic drying than with freeze drying is well established using nylon bag techniques (Mahyuddin et al., 1988; Ahn et al., 1989; Palmer and Schlink, 1992; Balogun et al., 1998). Our results, using an in vitro technique, show that the major decline in IVDMD occurred above 45°C. However, in this study IVDMD at all oven drying temperatures was similar to or substantially higher than the value for freeze-dried material. In the presence of PEG, the CIVDMD for anaerobically dried samples was similar to that for freeze-dried material. This may suggest that the CT in the freeze-dried material was more active in suppressing digestibility than that of the tannins in the oven-dried material.

The absence of response to temperature or drying condition with IVND was unexpected. Furthermore, the values were similar to the freeze-dried results. IVND is not usually measured, so we have no comparative data. It appears to be a useful modification of the Tilley and Terry (1963) technique for use with tanniniferous species (Jones et al., 2000). It could also be incorporated into techniques for the measurement of gas production to provide more information with tanniniferous species. Since the measurement of IVND is so important with tanniniferous feeds, the absence of any effect of drying method and temperature on its assessment should be studied further to see if this applies across species.

4.4. PEG binding

The absence of a response to increasing temperature of drying with PEG_BS for samples dried anaerobically or aerobically supports the findings of Silanikove et al. (1996) with aerobic drying. Our data, however, extend the range beyond the 50 and 90°C studied by these authors. However, values were appreciably lower than for freeze-dried samples. Comparisons between freeze-dried and oven-dried samples cannot therefore be made with any confidence.

The lower values for PEG_BR are in accord with other results from a range of tanniniferous browse species (Jones and Palmer, 2000). The reasons for these lower values are not clear, since exposure of the samples to labelled PEG were for longer duration and at a higher temperature, factors which should have increased PEG uptake (Silanikove et al., 1996). The loss of material through digestion during Stage 1 may be involved in the lower values recorded. Some support for this hypothesis was the higher PEG_BR for *C. calothyrsus* and *Acacia boliviana* that lost less DM after Stage 1 than did *Leucaena leucocephala* (Jones and Palmer, 2000). The presence of colonising bacteria on the plant material may also have impeded uptake of the PEG. The somewhat lower pH of
the digestion buffer medium than of the equilibration buffer (6.8 vs 7.1) and that it was anaerobic would appear to be an unlikely explanation.

The high PEG_BR value for samples dried aerobically at 25°C is unexplained, since at all other temperatures the levels for aerobic drying are lower than for anaerobic drying, and all were lower than the PEG_BR for the freeze-dried samples. This shows that oven drying has reduced the access to, or binding of, PEG to certain tannin fractions.

These data support the hypothesis that the degree of complexing of CT with components of the plant is affected by both temperature and oxidation, the latter having the greater effect particularly at high drying temperatures. At these high temperatures, CT may well covalently bond with cell components making them less available for digestion. For these reasons, it is clear that comparison of results across drying temperatures cannot be made with any confidence, except possibly at the lower drying temperatures where many results were similar to those from freeze-dried samples.

Drying samples in a stream of nitrogen to maintain anaerobic conditions was certainly effective in reducing the effects of drying temperatures. For many situations, however, this method may not be very practical. We conclude that in the absence of freeze drying facilities, samples should be dried at 45°C to minimise the adverse effects of drying on measures of the fibre fractions and of in vitro digestibility. At this temperature, there was little difference between aerobic and anaerobic drying for the various characteristics measured.

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