In vitro digestion studies using $^{14}$C-labelled polyethylene glycol (PEG): the effect of sample pretreatment on dry matter and nitrogen digestibility as well as PEG binding of *Calliandra calothyrsus*

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Abstract

The effect of sample preparation on measures of in vitro digestibility was studied using leaves of *Calliandra calothyrsus*. They were chopped fresh (FrCh), chopped and oven-dried at 65°C (ChOD), chopped and freeze-dried (ChFD), oven-dried and ground (ODGr) or freeze-dried and ground (FDGr). Samples were analysed for in vitro dry matter and nitrogen digestibility with or without PEG addition to bind tannins. Ground samples gave higher dry matter digestibilities (IVDMD) than chopped samples, freeze-dried samples had higher IVDMD than oven-dried samples, and fresh leaves were intermediate. The IVDMD values for FrCh, ChOD, ChFD, ODGr and FDGr were 45.3, 34.5, 40.5, 48.4 and 50.1%, respectively. Overall, PEG increased IVDMD from 42.1 to 45.4 ($P < 0.01$), and nitrogen digestibility (IVND) from 37.0 to 66.4% ($P < 0.01$). When corrected for PEG bound in the residue, CIVDMD increased by 8.6–15.5% depending on the pretreatment. PEG improved IVND of all samples, although the improvement expressed in percentage units was greatest with the ground samples and least with the dried chopped and fresh samples. The range in IVND was from 27.2% with ChOD–PEG to 86.0% with FDGr + PEG. The relation between PEG binding and IVND for the dried samples was linear ($r^2 = 0.96$), indicating that pretreatment influenced the ability of PEG to penetrate and bind to tannins. Results emphasise the importance of standardisation of sample pretreatment for PEG binding studies and again show that IVDMD studies with tanniniferous forage and browse species using PEG will give unreliable results because of the PEG-tannin complexes in the residue. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: In vitro digestibility; Tannins; PEG-binding; DMD; N-digestibility; Sample preparation
1. Introduction

In the evaluation of tropical feeds, in vitro digestion studies are commonly used to evaluate nutritive value. With tanniniferous feeds, PEG can be used to overcome the deleterious effects of condensed tannins. In an earlier study, (Palmer and Jones, 2000) it was shown that polyethylene glycol (PEG 4000) increased in vitro dry matter digestibility (IVDMD) and especially N-digestibility (IVND) of freeze-dried ground samples of the tropical leguminous browse shrub Calliandra calothyrsus. This was attributed to the binding with tannins to form PEG-tannin complexes. These were found to be insoluble in the acid pepsin of the stage-2 digestion and therefore remained in the residue. As a result, IVDMD was underestimated. It is probable that the accessibility of any condensed tannin to the PEG could be influenced by the particle size of the feed. In addition, the access of bacteria to the substrate could also be affected by sample pretreatment.

In this study, the effect of sample pretreatment on both IVDMD and IVND in the presence and absence of PEG was assessed. PEG binding was also measured using 14C-PEG and corrections were made to IVDMD to give a corrected value (CIVDMD).

2. Materials and methods

Leaf samples (≈4% N in DM) were obtained from well-established Calliandra calothyrsus (Commonwealth Plant Introduction Number, Australia (CPI) 115690) shrubs, growing under irrigation in rows 3 m apart at the CSIRO Lansdown Research Station 50 km south of Townsville, in north Queensland, Australia (19°40′S, 146°50′E).

The five pretreatments on samples consisting of the terminal five fully expanded leaves of actively growing shoots, were:

1. fresh leaves chopped with a rolling knife to ≈3 mm lengths (FrCh);
2. leaves chopped as above, then oven-dried at 65°C (ChOD);
3. leaves chopped as above, then freeze-dried (ChFD);
4. leaves dried at 65°C, then grounded to pass a 1 mm screen (ODGr);
5. leaves freeze-dried and then ground to pass a 1 mm screen (FDGr).

In vitro digestion studies were conducted on the samples using a modified Tilley and Terry (1963) two-stage digestion method (Palmer and Jones, 2000). The equivalent of 0.4 g DM of each sample was weighed into glass centrifuge tubes. Dried samples received 0.6 ml water to balance the water content of the fresh sample. Each tube then received 2 ml of distilled water or 2 ml of PEG 4000 solution (32 g/l spiked with 14C PEG 4000, Amersham, UK), followed by 40 ml of rumen fluid/buffer (1:4). There were two replicates of each treatment together with four blank tubes that received no plant sample, giving 24 tubes in total.

Tubes were incubated in an anaerobic chamber at 39°C for 72 h, then centrifuged for 20 min at 2500 g. Two samples, each of 1 ml, were withdrawn from the supernatant and added to 10 ml of liquid scintillator (OptiPhase ‘HiSafe’; Fisher Chemicals, England) for counting radioactivity. The remaining supernatant was decanted and 40 ml of distilled water at 39°C was added. Tubes were thoroughly mixed on a vortex mixer and then
centrifuged for 20 min, the supernatant again discarded, and the process repeated. Each tube then received 40 ml of acid pepsin (2 g of 1 : 10,000 pepsin in 1 l of 0.1 M HCl); contents were thoroughly mixed on the vortex mixer and then incubated at 39°C for 24 h. Tubes were then centrifuged for 20 min, duplicate samples of the supernatant were withdrawn for counting, the remaining supernatant was discarded, and the residue dried at 80°C for 48 h prior to weighing. IVDMD was calculated from the difference between the initial dry sample and the dry residue minus the mean of the residue in the blank tubes. IVND was calculated from the difference between the initial N in the sample and the N in the residue minus the mean N of the residue in the blank tubes.

Radioactivity of the supernatant samples was counted on a β-scintillation counter (Wallac 1410 Pharmacia, Finland). These counts enabled calculation of the amount of PEG bound in the residue after stage-1 digestion or released during stage-2 in acid pepsin. A corrected estimate of IVDMD (CIVDMD) could then be made by subtracting the amount of bound PEG from the residue weights before calculations were made. No such correction was necessary for estimating IVND.

Treatment effects were analysed using ANOVA. The IVDMD, CIVDMD and IVND values were regressed on the values for PEG binding, as were the differences between the ‘+’ and ‘−’ PEG treatments on N and DM digestibilities.

3. Results

Pretreatment influenced (P < 0.01) IVDMD (Table 1). Among the dried samples, chopping gave much lower values than the grinding (P < 0.01). With values for the fresh chopped leaf intermediate, oven-dried (OD) chopped samples gave lower values than the freeze-dried (FD) chopped leaves (P < 0.01). Similar results were obtained for the

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IVDMD (%)</th>
<th>CIVDMD (%)</th>
<th>IVND (%)</th>
<th>PEG bound (mg/g DM)</th>
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<tr>
<td>FrCha</td>
<td>41.7</td>
<td>na</td>
<td>31.5</td>
<td>na</td>
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<tr>
<td>ChOD</td>
<td>33.6</td>
<td>na</td>
<td>27.2</td>
<td>na</td>
</tr>
<tr>
<td>ChFD</td>
<td>39.8</td>
<td>na</td>
<td>30.3</td>
<td>na</td>
</tr>
<tr>
<td>ODGr</td>
<td>47.8</td>
<td>na</td>
<td>45.8</td>
<td>na</td>
</tr>
<tr>
<td>FDGr</td>
<td>47.8</td>
<td>na</td>
<td>50.1</td>
<td>na</td>
</tr>
<tr>
<td>FrCh + PEG</td>
<td>48.9</td>
<td>56.2</td>
<td>59.6</td>
<td>73.1</td>
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<tr>
<td>ChOD + PEG</td>
<td>35.5</td>
<td>42.2</td>
<td>45.8</td>
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</tr>
<tr>
<td>ChFD + PEG</td>
<td>41.2</td>
<td>49.8</td>
<td>57.0</td>
<td>86.4</td>
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<td>ODGr + PEG</td>
<td>49.2</td>
<td>60.5</td>
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<tr>
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<td>86.0</td>
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<td>LSD (P &lt; 0.05)</td>
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<td>LSD (P &lt; 0.01)</td>
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<td>5.06</td>
<td>5.21</td>
<td>9.7</td>
</tr>
</tbody>
</table>

Table 1
The effect of sample pretreatment of Calliandra calothyrsus leaves on in vitro dry matter digestibility (IVDMD), PEG corrected IVDMD (CIVDMD), in vitro nitrogen digestibility (IVND) and PEG binding

*a Fr: fresh, OD: oven dried, FD: freeze dried, Ch: chopped to ≈3 mm lengths, Gr: ground through a 1 mm screen.
b n.a.: Not applicable.
ground leaves. The mean of the FD treatments (both chopped and ground) had a similar value to the fresh chopped leaf (45.3 versus 45.2%). Overall, PEG increased IVDMD from 42.1 to 45.4% \((P < 0.01)\) and CIVDMD from 42.1 to 54.4% \((P < 0.01)\). There was no statistically significant PEG pretreatment interaction \((P > 0.05)\). Among the PEG treatments, the CIVDMD values ranked from lowest to highest: ChOD, ChFD, FrCh, ODGr and FDGr. This ranking was the same as for the uncorrected samples (Table 1).

The effects of both PEG and pretreatment had greater effects on IVND than on IVDMD (Table 1). The interaction was also significant \((P < 0.01)\). Although PEG increased IVND of all treatments, the effect on the ground samples was greater than for the chopped samples. IVND of the fresh chopped leaf was intermediate between the dried chopped and dried ground treatments without and with PEG. However, the improvement due to PEG was similar to that of the dried chopped treatments (Table 1).

The level of PEG binding was highest with the grounded samples (>100 mg/g DM) and lowest with the OD chopped samples (66.4 mg/g DM). The ChFD samples gave higher PEG binding than the ChOD samples, whereas the fresh chopped leaf gave intermediate values (Table 1).

For the dried samples, there was a linear relation between IVDMD and PEG bound to the residue \((r^2 = 0.99)\). The relation between CIVDMD and PEG bound for the dried samples was also linear \((r^2 = 0.96)\); as was the relation between the IVND and the PEG bound values for dried samples \((r^2 = 0.96)\) (Fig. 1). The fresh samples gave higher than

![Fig. 1. The relation between increasing levels of bound PEG due to different sample pretreatments and: in vitro dry matter digestibility (IVDMD), (■); PEG corrected IVDMD (CIVDMD), (▲); and in vitro nitrogen digestibility (IVND), (●); for samples of C. calothyrsus leaf material given different pretreatments prior to in vitro digestion (values for FrCh (open symbols) not included in the regressions). The regressions are described by the equations: (■) \( y = 12.8 + 0.313x \) \((r^2 = 0.99)\); (▲) \( y = 12.9 + 0.438x \) \((r^2 = 0.96)\); (●) \( y = -15.4 + 0.899x \) \((r^2 = 0.96)\). Fr: fresh, OD: oven dried, FD: freeze dried, Ch: chopped to \(\approx3\) mm lengths, Gr: grounded through a 1 mm screen.](image-url)
the predicted values for IVDMD, CIVDMD and IVND for its PEG binding value of 73 mg/g DM.

The improvement in IVND of the samples with PEG was linearly related to the PEG binding values (Fig. 2). The response to freeze drying and grinding treatments being greater than for the chopped and oven-dried material. There was however no significant relationship in DMD improvement due to PEG with increased PEG binding.

4. Discussion

Sample preparation clearly influenced all parameters measured. It seems apparent from the PEG binding data that tannins were a major factor in the treatment effects measured. The ground samples had the highest PEG binding and so had the greatest opportunity to overcome negative effects of tannins on both IVDMD and IVND. Consequently, the improvement in IVND was greater for these samples. It was noted that ChFD samples were more brittle and tended to break up more than the ChOD samples and this may have reduced particle size and increased the surface area. Differences between the ground OD and FD samples were also apparent. Clearly, in vitro digestibilities cannot be estimated on intact plant material, yet with a forage such as *Calliandra* any treatment to increase access of bacteria to plant cells will affect the digestion parameters being measured.
Tannins were not the only factor involved, since in the absence of PEG, there were also effects of sample pretreatment on both IVND and to a lesser extent on IVDMD. This may be associated with smaller particle size and better access to plant material by the rumen bacteria as shown in other studies using nylon bags (Chapman and Norton, 1982; Shaver et al., 1984). Although coarse or fine grinding had little effect on IVDMD with temperate forages, ball-milling increased IVDMD (Tilley and Terry, 1963). This is considered to be associated with greater surface area allowing rapid access by bacterial enzymes. With freeze-dried samples, grinding to pass a 1 mm screen may be adequate for maximum PEG effect since with ball milling of these samples there was only a small increase in the values for PEG binding (≈6%) (Jones and Palmer, 2000). The same may not be true for oven-dried material.

The low IVDMD of the fresh sample contrasts with the high digestibility of similar material in dacron bags in the rumen of fistulated cattle (Palmer and Schlink, 1992; Balogun et al., 1998). Both these studies recorded DM losses of about 80% in the absence of any PEG, however, whole tract dry matter digestibilities in sheep fed fresh leaves of *C. calothysus* have given similar low results (B. Palmer, unpublished). One possible explanation for the higher dacron bag digestibility values is the lack of any feed-back of the adverse effects of tannin in the rumen compared with that of the closed in vitro system. It is known that tannins in *Calliandra calothyrsus* are inhibitory to some rumen bacteria (Mwendia, 1994; McSweeney et al., 1998), giving some credence to this hypothesis.

The PEG bound in the residue after stage-1 digestion was not released after acid-pepsin digestion and so remained in the final residue. This agrees with our earlier studies (Palmer and Jones, 2000) and with the predictions of Makkar et al. (1995), based on their results with neutral detergent and acid detergent solubles. This PEG retention will result in a higher residue weight and hence a lower estimated IVDMD. Failure to account for this tannin effect will give erroneous predictions of IVDMD, when feeds contain condensed tannins. The use of 14C-PEG enables the ‘true’ IVDMD to be calculated. However, in many situations the measurement of IVND may be a more appropriate option since the only additional measurement to the Tilley and Terry (1963) IVDMD is the N analysis on the residues and on the feed. This latter value is often measured routinely on feeds that are subsequently analysed for IVDMD.

In view of the large differences in digestibility in vitro due to particle size of the feed, it is important to standardise methodologies, so that reliable comparisons can be made. Freeze-dried ground samples resulted in the highest digestibility values and is therefore the recommended method. However, in the absence of freeze drying facilities, samples oven dried at 65°C and grounded through a 1 mm screen are also deemed acceptable.

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References


