A Revised Method of Condensed Tannin Analysis in Leucaena spp.

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ABSTRACT
The proanthocyanidin (butanol-HCl) assay was used to measure condensed tannin (CT) in extracts from *Leucaena* spp. at the University of Queensland. Inconsistent results were found to be caused by the iron catalyst in the butanol/HCl reagent and the presence of ascorbic acid in the sample matrix which enhanced anthocyanidin development. In addition, preparation of sample extracts by back extraction with organic solvents reduced CT recoveries. An accurate and rapid technique was developed that measures CT directly in 70% aqueous acetone 0.1% (w/v) sodium metabisulphite plant extracts.

KEYWORDS
Condensed tannin, proanthocyanidin assay, sample preparation, *Leucaena*

INTRODUCTION

Condensed tannins (CT) in plant material are best quantified by extraction with 70% aqueous acetone (Cork and Krockenberger, 1991), followed by the proanthocyanidin (butanol/HCl) assay. This assay measures anthocyanidins produced by CT acid depolymerisation, which Porter et al. (1986) optimized by adding a ferric iron catalyst to the reagent. University of Queensland studies using this optimized assay resulted in inconsistent estimations of CT concentrations in the *Leucaena* genus.

Pigments present in crude 70% aqueous acetone plant extracts have been hypothesised to interfere in colorimetric assays for CT measurement. Perez-Maldonado and Norton (1996) and Terrill et al. (1992) recommended back extraction of samples with organic solvents to remove pigments. These procedures are tedious and a number of authors have proposed assaying crude extracts directly (Hagerman, 1995). Antioxidants, such as ascorbic acid or sodium metabisulphite, are normally added to extraction solvents to protect CT from oxidation but their effects on the proanthocyanidin assay have not been evaluated.

The sensitivity of the proanthocyanidin assay to the presence of antioxidants and iron (Fe^{3+}) was investigated to identify the source of inconsistency. Recovery tests, in which CT was added to *Leucaena* spp. leaf material, were conducted to evaluate the effects of a) different sample preparation procedures b) the presence or absence of antioxidants in crude plant extracts, on the accuracy of CT measurement.

MATERIALS AND METHODS

Leaf sample preparation. Youngest fully expanded leaves of *Leucaena leucocephala* (de Wit Lam) cv. Tarramba (K636) and *L. pallida* (Britton and Rose) (CQ3439) were harvested at Redland Bay (27°37'S:153°19'E), Queensland, Australia, and immediately frozen in dry ice. Leaves were lyophilized, ground to pass a 1-mm sieve and stored at 4°C.

Proanthocyanidin assay evaluation. CT standards purified from *L. leucocephala* cv. Tarramba and *L. pallida* (K376) leaves, prepared by Sephadex LH-20 adsorption chromatography, were subjected to the following butanol/HCl treatments:

(i) Fe^{3+} concentrations of 0-50 μg mL^{-1} in the final sample/reagent matrix prepared by adding ferric ammonium sulphate ($FeNH_4(SO_4)_2.12H_2O$) dissolved in 0.15 mL of 0.1 M HCl to 400 μg of CT dissolved in 1 mL of 68% aqueous methanol.
(ii) Ascorbic acid concentrations in the final sample/reagent matrix of 0-400 μg mL^{-1} prepared in 1 mL of 68% aqueous methanol, containing 400 μg of CT.
(iii) Sodium metabisulphite ($Na_2S_2O_5$) concentrations of 0-263 μg mL^{-1} in the final sample/reagent matrix, prepared in 1 mL of 70% aqueous acetone containing 400 μg of CT.

In all cases, 5 mL of 95% butan-1-ol/HCl reagent was added, the tubes sealed and incubated in a 95°C waterbath for 60 minutes. Colour development was measured at 550 nm.

Recovery tests. Purified CT from *L. leucocephala* was added to 200 mg leaf samples prior to extraction by 4 sequential 20 minute sonications in 10 mL of 70% aqueous acetone. Recoveries were calculated from the difference between the CT content of spiked and unspiked control extractions.

Back extraction. Acetone extracts containing 0.1% ascorbic acid were back extracted with diethyl ether and ethyl acetate (Perez-Maldonado and Norton, 1996), then adjusted to 68% methanol prior to colour development.

Antioxidant effects. The effects of pigments and antioxidants on the measurement of CT directly in 70% acetone extracts were evaluated. Spiked and unspiked samples were assayed in 1 mL of crude extract following the procedure of Hagerman (1995), but with the omission of the iron catalyst from the butanol/HCl reagent. Recovery tests were also carried out 1 mL on 70% acetone extracts containing 0.1% $Na_2S_2O_5$ as an alternate antioxidant.

RESULTS AND DISCUSSION

Proanthocyanidin assay. *Iron effects.* The presence of Fe^{3+} in the butanol/HCl reagent initially increased the colour yield from *Leucaena* spp. CT (Figure 1a), however at concentrations >15 μg mL^{-1} absorbance was suppressed due either to inhibition of acid depolymerisation of CTs or to degradation of anthocyanidins. This contrasts with the findings of Porter et al. (1986) who reported a plateau in anthocyanidin yield from *Chaenomeles speciosa* CT with increasing Fe^{3+} concentration. This problem is exacerbated by the degradation of Fe^{3+} salt on storage, preventing consistent reagent preparation in the narrow optimal range of 4-15 μg mL^{-1} Fe^{3+} in the sample/reagent matrix. Fe^{3+} was excluded from the butanol/HCI reagent to improve the reproducibility of colour development from *Leucaena* spp. CT, supporting the observations of Terrill et al. (1992).

Antioxidants. Increasing ascorbic acid concentration in 68% methanol extracts enhanced colour development, reaching a plateau at sample/reagent matrix concentrations ž200 ug mL^{-1} (Figure 1b). *L. leucocephala* CT (+60%) was more sensitive than *L. pallida* CT (+20%). Ascorbic acid may catalyse CT depolymerisation or enhance the absorbance of anthocyanidins at 550 nm. Consequently, ascorbic acid must be added to the reference CT standards at the same concentration present in the samples to ensure accurate CT determination.

The 70% acetone sample matrix, without antioxidant, produced erratic results in the proanthocyanidin assay. At low sample CT

concentrations (<400-500 µg mL^{-1}) colour development was completely inhibited (data not presented). Acetone extracts containing 0.1% (w/v) ascorbic acid also inhibited colour development (data not presented). Addition of $Na_2S_2O_5$ to the extraction solvent as an alternative antioxidant, above threshold concentrations of 57 and 76 µg mL^{-1} $Na_2S_2O_5$ in the final sample/reagent matrix for *L. pallida* and *L. leucocephala* CT respectively, resulted in stable colour development over a wide range of CT concentrations (data not presented). For convenience, we adopted 0.1% (w/v) $Na_2S_2O_5$ 70% acetone as the extraction solvent, producing a concentration of 167 µg mL^{-1} $Na_2S_2O_5$ in the sample/reagent matrix.

The proanthocyanidin assay is sensitive to the presence of catalysts, antioxidants and the CT solvent matrix and requires modification to suit the sample preparation technique and type of CT under investigation.

Recovery tests. *Back extraction techniques.* The method of Perez-Maldonado and Norton (1996) resulted in poor recoveries (83-91%), with CT losses observed in ethyl acetate back extractions (Table 1). *Crude extract analysis.* The measurement of CT in 70% acetone extracts in the absence of Fe^{3+} and antioxidants, resulted in significant overestimation with recoveries of added CT levels ranging from 118-162% (Table 1). CT cannot be reliably measured in these extracts due to the inexplicable suppression of colour at low CT concentrations. However, recoveries from acetone extracts containing 0.1% $Na_2S_2O_5$ ranged from 91-108% for both *Leucaena* spp. indicating that CT can be accurately measured directly in acetone extracts containing this antioxidant. Standard curves obtained using this modified solvent were consistently linear with a high degree of correlation (r^2=0.999) over the range 25-1250 µg mL^{-1} CT, in contrast to the incongruous biphasic relationship reported by Porter et al. (1986).

The modified method of CT analysis described in this paper is accurate, simple and rapid, and is currently being used to investigate genotypic and agronomic variation in CT in the *Leucaena* genus.

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Table 1
Recovery of extractable CT added to Lyophilized *Leucaena* spp. leaf material subjected to different sample preparation procedures and the presence or absence of an antioxidant in the extractant.

Leaf Material	Added CT (mg)	Recovery of extractable CT (%)		
		Back extracted 70% aqueous acetone extracts samples[1]		
			no antioxidant[2]	+ $Na_2S_2O_5$
L. leucocephala (K636)	10	87 aA†	155 bA	108 cA
	20	91 aA	118 bB	100 cB
L. pallida (CQ3439)	20	92 aA	162 bA	102 cA¶
	40	83 aB	158 bA	91 aB¶

1 Perez-Maldonado and Norton (1996)
2 modification of Hagerman (1995)
† means within rows followed by the same lower case letter are not significantly different (P<0.05). Means within columns for each separate species followed by the same capital letter are not significantly different (P<0.05)
¶ NOTE: leaf material spiked with *L. pallida* (K376) CT

Figure 1
Anthocyanidin yield expressed as absorbance at 550 nm of *L. leucocephala* (K636) º and *L. pallida* (K376) Ð CT, at varying (a) Fe^{3+} and (b) ascorbic acid concentrations in the final sample/reagent matrix.