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Limitation of the butanol–hydrochloric acid–iron assay for bound condensed tannins

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Abstract

The butanol–HCl–iron method is widely used for measurement of extractable condensed tannins (*syn.* proanthocyanidins) in foods and feeds. As the method is based on acid catalysed oxidative depolymerization of condensed tannins into anthocyanidins, this method has also been used for determination of bound condensed tannins. The recovery of bound condensed tannins by the butanol–HCl–iron assay was monitored by subjecting the residues left after the assay to solid-state ^{13}C NMR spectroscopy. The signal at δ 155.0, indicative of condensed tannins, remained relatively high in the residues following the butanol–HCl–iron assay, suggesting an incomplete recovery of bound condensed tannins by the assay. The results obtained using the butanol–HCl–iron assay should therefore be interpreted with caution. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Condensed tannins; Butanol–HCl assay; NMR spectroscopy; Bound proanthocyanidins

1. Introduction

Condensed tannins or proanthocyanidins are flavanol based polymers occuring widely in the Plant Kingdom and are known to play various nutritional and ecological roles (Hagerman, Zhao, & Johnson, 1997; Makkar, 1993; Mueller-Harvey & McAllan, 1992). These are generally quantified by the vanillin–HCl methods (Broadhurst & Jones, 1978; Price, Van Scoyoc, & Butler, 1978) and the *n*-butanol–HCl–iron method (Bate-Smith, 1973; Porter, Hrstich, & Chan, 1986). The latter is more specific and depends on the oxidative depolymerization of the inter-flavan bonds under hot acidic conditions to give anthocyanidins which are measured spectrophotometric-ally at 550 nm (Hagerman et al., 1997). In addition to measurement of extractable condensed tannins, the butanol–HCl–iron method has also been widely used for measurement of bound condensed tannins (Degen, Becker, Makkar, & Borowy, 1995; Makkar, Becker, & Younan, 1997; Makkar, Bluemmel, & Becker, 1997; Makkar & Singh, 1991; Reed, 1986; Terrill, Rowan, Douglas, & Barry, 1992). The use of this assay for measurement of bound condensed tannins assumes that the bound condensed tannins are quantitatively released from the sample during the assay. The present paper is an attempt to verify this assumption. In order to monitor the extractability of tannins by the butanol–HCl–iron reagent, solid-state ^{13}C nuclear magnetic resonance (NMR) spectroscopy was used.

2. Materials and methods

2.1. Leaf samples

Four tannin-rich (*Acacia saligna*, *Robinia pseudoacacia*, *Eugenia jambolana* and *Eucalyptus punctata*) and one tannin-free (white clover) leaf samples were lyophilized. These leaf samples and their neutral detergent fiber (NDF) fractions were used for the experiment. The NDF was prepared essentially as described by Van Soest, Robertson, and Lewis (1991) except that the NDF was dried using a lyophilizer (Makkar & Singh, 1995).

2.2. Condensed tannin analysis

These were determined essentially by the method of Porter et al. (1986). In brief, 10 mg sample (leaf or

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NDF) was weighed in a test tube and to it were added 0.5 ml of 70% aqueous acetone (v/v), 3 ml of *n*-butanol–HCl solution (95:5, v/v) and 0.1 ml of the iron reagent (2% ferric ammonium sulfate in 2 N HCl). This reaction could have been carried without the addition of 0.5 ml of 70% aqueous acetone. Since the extractable tannins are generally extracted in 70% aqueous acetone, the standard procedure of Porter et al. was adopted to facilitate comparison of the values obtained with that of the extractable condensed tannins, if needed. The test tubes were covered with glass marbles and heated at 95°C for 1 h using a heating block. The test tubes were cooled to room temperature, centrifuged and absorbance measured at 550 nm. This absorbance has been designated as 'standard absorbance' to describe absorbance measured by the standard procedure (Fig. 1). The residue left after centrifugation was washed twice, each time with 3 ml of butanol followed by centrifugation. The washed residue from several tubes was pooled and lyophilized (residue×1). A portion of this residue was subjected to NMR spectroscopy and also to condensed tannin determination using the butanol–HCl–iron reagent by taking 10 mg of the residue. This spectrum was designated as spectrum×1 and absorbance observed after the second treatment of the sample with the butanol–HCl–iron reagent as absorbance×1. The residue was again washed with butanol as described above and the washed residue was pooled (residue×2) and again subjected to measurement of condensed tannins (absorbance ×2). Similarly residues×3 and ×4 were obtained and absorbance×3 was recorded. The residue×4 obtained after a total of four treatments of the sample with the butanol–HCl–iron reagent and after washing with butanol and lyophilization was also subjected to NMR spectroscopy. When the absorbance was >0.6, appropriate dilution was made with the butanol–HCl reagent before remeasuring the absorbance at 550 nm. A similar treatment was performed using micro-crystalline cellulose as the sole substrate along with an internal standard hexatriacontane, which was unaffected by the acid treatment.

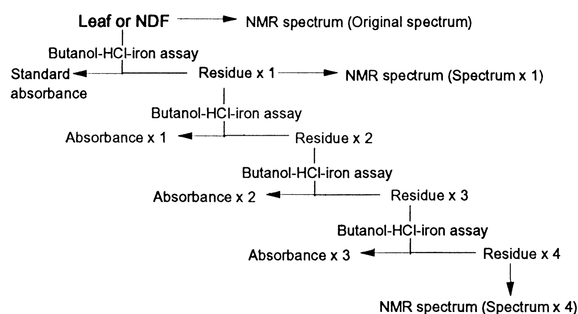

Fig. 1. Schematic presentation of sample preparation for spectrophotometric and spectroscopic measurements

2.3. NMR spectroscopy

The leaf and NDF samples and residues×1 and ×4 were subjected to ^{13}C cross-polarization and magic angle spinning (CPMAS) NMR spectroscopy as described earlier (Gamble, Akin, Makkar, & Becker, 1966).

3. Results and discussion

The solid-state ^{13}C NMR spectra of *A. saligna* leaf and NDF samples are shown in Figs. 2 and 3. Similar spectra were observed for three other tannin-rich samples (not shown). The spectral peak heights of resonances representative of chemical constituents are shown in Table 1. These values have been normalized to the crystalline cellulose peak at (δ 89.0 whose intensity is arbitrarily set to 2.0. This assumes that the crystalline cellulose is unaffected by the treatment, which was borne out in the control experiment with micro-crystalline cellulose. The peak at δ 155.0 is indicative of condensed

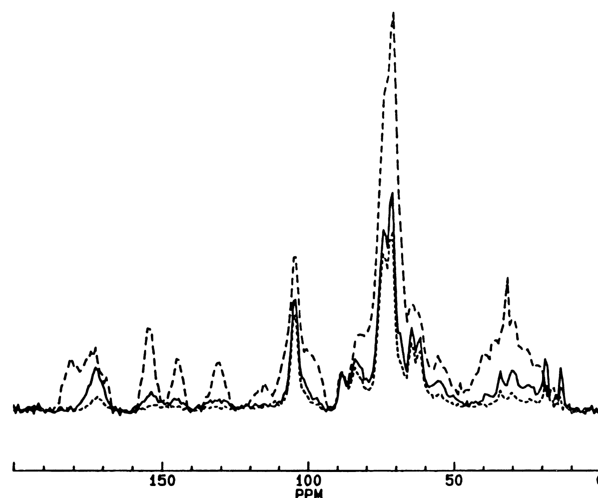

Fig. 2. CPMAS ^{13}C NMR spectra of *Acacia saligna* leaf (– – original, ____, ×1, - - ×4).

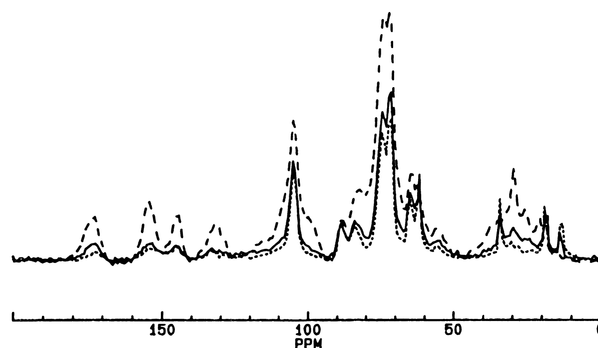

Fig. 3. CPMAS ^{13}C NMR spectra of *Acacia saligna* neutral detergent fiber (– – original, ____, ×1, - - ×4).

Table 1
^{13}C CPMAS NMR spectra peak heights (cm) of resonances representative of chemical constituents, normalized to the crystalline cellulose peak at δ 89.0[a]

	Wax (δ 182.0)	Protein (δ 174.0)	Condensed tannin (δ 155.0)	Cellulose (δ 89.0)	Pectin (δ 55.0)
Acacia saligna					
Leaf					
Original spectrum	2.5	2.3	3.6	2.0	2.0
Spectrum (×1)	0.0	1.8	0.9 (25.0)	2.0	1.2
Spectrum (×4)	0.0	0.7	0.3 (8.3)	2.0	0.7
NDF					
Original spectrum	0.0	1.9	2.6	2.0	1.3
Spectrum (×1)	0.0	0.9	0.9 (34.6)	2.0	1.0
Spectrum (×4)	0.0	0.7	0.7 (26.9)	2.0	0.7
Robinia pseudoacacia					
Leaf					
Original spectrum	3.0	8.4	3.4	2.0	5.8
Spectrum (×1)	0.0	3.2	0.8 (23.5)	2.0	3.2
Spectrum (×4)	0.0	1.3	0.0	2.0	1.3
NDF					
Original spectrum	0.0	6.3	1.8	2.0	4.3
Spectrum (×1)	0.0	3.2	0.8 (44.4)	2.0	2.0
Spectrum (×4)	0.0	1.8	0.8 (44.4)	2.0	1.3
Eugenia jambolana					
Leaf					
Original spectrum	0.5	1.3	1.7	2.0	1.0
Spectrum (×1)	0.0	1.0	0.4 (23.5)	2.0	1.0
Spectrum (×4)	0.0	0.4	0.2 (11.8)	2.0	1.0
NDF					
Original spectrum	0.0	1.0	1.1	2.0	0.7
Spectrum (×1)	0.0	0.4	0.4 (36.4)	2.0	0.6
Spectrum (×4)	0.0	0.1	0.4 (36.4)	2.0	0.5
Eucalyptus punctata					
Leaf					
Original spectrum	0.0	5.8	5.0	2.0	—
Spectrum (×1)	0.0	1.3	0.5 (10.0)	2.0	1.0
Spectrum (×4)	0.0	0.5	0.3 (6.0)	2.0	0.5
NDF					
Original spectrum	0.0	2.8	1.8	2.0	—
Spectrum (×1)	0.0	1.0	0.5 (27.8)	2.0	1.0
Spectrum (×4)	0.0	0.5	0.5 (27.8)	2.0	0.8
White clover					
Leaf					
Original spectrum	0.0	8.5	0	2.0	6.0
Spectrum (×1)	0.0	3.3	0	2.0	1.8
NDF					
Original spectrum	0.0	3.3	0	2.0	2.5
Spectrum (×1)	0.0	0.5	0	2.0	0.3

[a] Values in parentheses are peak heights relative to that of original spectrum, which was taken as 100%.

tannins (Gamble et al., 1996). White clover is known to be free of tannins, and in accordance with this the signal at δ 155.0 was absent (Table 1) and so was the absorbance at 550 nm (Table 2). The spectral intensities at δ 155.0 of the residues (×1) were 25, 23.5, 23.5 and 10% of the original intensity of the corresponding leaf samples of *A. saligna*, *E. jambolana*, *R. pseudoacacica*, and *E. punctata*, respectively. These values for the NDF samples were even higher: 34.6, 36.4, 44.4 and 27.8%, respectively. A substantial signal was also observed at δ 155.0 in the residues obtained after four-time treatment with the butanol–HCl–iron reagent (Table 1). These results suggest that substantial amounts of condensed tannins were present in the residues following the standard butanol–HCl–iron procedure, the extent of remaining condensed tannins being higher in the NDF samples. In addition, similar peak heights at δ 155.0 of residues×1 and ×4 for NDF samples of *E. jambolana*, *R. pseudoacacica*, and *E. punctata* and a small decrease in the peak height on subjecting *A. saligna* NDF to the butanol–HCl–iron assay four times (Table 1) show that the repeated use of butanol–HCl–iron assay does not increase the extractability of condensed tannins.

Table 2
Absorbance (550 nm) of leaf and neutral detergent fiber (NDF) samples[a]

	Absorbance			
	Standard	×1	×2	×3
Acacia saligna				
Leaf	13.1 ± 1.04	0.43 ± 0.026	0.19 ± 0.006	0.09 ± 0.008
NDF	13.9 ± 1.01	1.04 ± 0.05	0.57 ± 0.085	0.22 ± 0.079
Robinia pseudoacacia				
Leaf	8.82 ± 1.26	0.17 ± 0.006	0.14 ± 0.025	0.12 ± 0.008
NDF	1.8 ± 0.44	0.24 ± 0.026	0.20 ± 0.046	0.15 ± 0.021
Eugenia jambolana				
Leaf	8.2 ± 0.72	0.16 ± 0.02	0.15 ± 0.016	0.07 ± 0.015
NDF	2.05 ± 0.13	0.18 ± 0.02	0.13 ± 0.012	0.09 ± 0.006
Eucalyptus punctata				
Leaf	2.78 ± 0.26	0.064 ± 0.003	0.064 ± 0.016	0.06 ± 0.006
NDF	1.86 ± 0.15	0.11 ± 0.02	0.091 ± 0.01	0.07 ± 0.005
White clover				
Leaf	0	0	0	0
NDF	0	0	0	0

[a] Values are mean ± SD ($n = 3$).

The relative absorbances for residues×1, ×2 and ×3 of leaf samples were from 0.7 to 3.3% of the standard absorbance (Table 2), suggesting that almost all the tannins which are extractable in the assay become extracted from the leaf sample during the first treatment with the butanol–HCl–iron reagent. On the other hand, the peak height peak at δ 155.0 of condensed tannins in the residue ×1 was from 10 to 25% of that of the leaf sample. Similarly, the relative absorbances for residue×1 of NDF samples, though higher than that for the corresponding leaf samples (Table 2), were substantially lower than the relative peak height of their spectrum ×1 (relative absorbances 6 to 13% and relative peak heights 27.8 to 44.4%). Similar pattern was observed for absorbance×3 and spectrum×4, suggesting lower extractability of condensed tannins from NDF

samples compared to leaf samples by the butanol–HCl–iron reagent.

The above results suggest that condensed tannins are not extracted quantitatively by the butanol–HCl–iron assay. The extractability of condensed tannins present in the bound form is poor in the assay. The butanol–HCl–iron method underestimates the levels of bound condensed tannins. Although the extent of under-estimation could be substantially higher for the NDF bound condensed tannins, the butanol–HCl–iron method does not appear to be suitable for measuring condensed tannins even in leaf samples. Limitations of this assay for measuring condensed tannins in faeces and digesta samples have also been highlighted by Terrill, Waghorn, Woolley, McNabb, and Barry (1994). Oven drying of samples also decrease recovery of condensed tannins bound to macromolecules (Makkar & Singh, 1991). The results obtained using the butanol–HCl–iron assay should be interpreted with caution, especially when this assay is used for measuring bound condensed tannins. The extraction condition (the butanol–HCl–iron reagent coupled with the heat treatment) used in this study is stronger than 70% aqueous acetone and the sodium dodecylsufate treatments used in the method of Terrill et al. (1992) and hence it is expected that the use of butanol–HCl reagent with or without the addition of iron will not release all the fiber-bound condensed tannins in the method of Terrill et al. (1992). The butanol–HCl–iron assay has been used extensively in our studies on the fate of fiber-bound condensed tannins in in vivo (Degen et al., 1995; Makkar, Borowy, Becker, & Degen, 1995) and the in vitro rumen true and apparent digestbilities of tannin-rich samples (Makkar et al., 1997). However, the conclusion drawn in these studies that the origin of higher absorbance in fiber fraction after fermentation is the soluble condensed tannins of the leaf sample which bind to proteins and appear in fiber fractions as artifacts is not affected by the results of this study. Since, in spite of incomplete extraction of bound condensed tannins by the assay, the fractions (NDF, acid detergent fiber and acid detergent lignin) obtained after fermentation had higher absolute absorbances as compared in the corresponding fractions before fermentation (Degen et al.; Makkar et al., 1995). Had the absolute absorbance in fiber fractions obtained after fermentation in in vitro (Makkar et al., 1997) or in the feces in in vivo experiments (Degen et al.; Makkar et al., 1995) been lower compared in the corresponding fraction before fermentation (which can occur depending on the nature and amount of tannins in a feed) this would have led to misleading conclusions.

A comparison of the solid-state ^{13}C NMR spectra of *A. saligna* leaf and NDF samples is shown in Fig. 4. The primary changes which occur upon treatment of the leaf sample with neutral detergent solution are the near quantitative loss of lipids, indicated by the decrease in

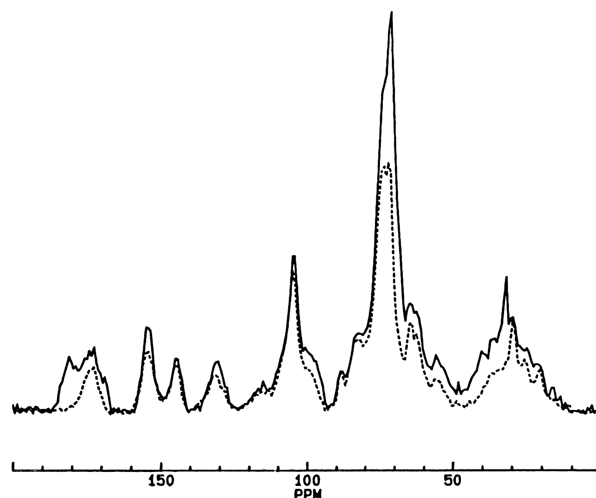

Fig. 4. CPMAS ^{13}C NMR spectra of *Acacia saligna* leaf (____) and neutral detergent fiber (- -).

the peak at δ 182, and the loss of approximately 35% of the pectins, as indicated by the decrease in the peak at δ 55. Pectins are considered to be soluble upon treatment with neutral detergent solution to obtain NDF (Van Soest, 1994), but a substantial signal was detected at δ 55 in the NDF samples (Table 1). It may be noted that the signal at δ 55 could also arise from the methoxy group present in hemicellulose rich in uronic acids. The protein fraction decreased by less than 20%, and the tannin fraction by approximately 28% upon NDF treatment of *A. saligna* leaf. Similar results were obtained with other tannin-rich leaf samples (Table 1). This resistance may be a result of the well known tendency of the two components to form strong complexes (Makkar, Becker, & Younan, 1997; Makkar et al., 1995).

In our earlier study on nutritional significance of bound condensed tannins, it was found that the bound condensed tannins become biologically active due to their release into the medium as a result of microbial action (Makkar, Becker, & Younan, 1997), and their presence in the bound form might affect fiber digestion (Van Soest, Conklin, & Horvath, 1986). These observations highlight the importance of accurate measurement of bound condensed tannins in feeds. Recently, a method based on depolymerization of proanthocyanidins in the presence of a nucleophile, toluene-α-thiol (thiolysis) and separation of degraded products using GC or HPLC has been developed for quantitative determination of condensed tannins (Matthews, Mila, Scalbert, Pollet et al., 1997). This method has been described to be more specific and sensitive compared to the butanol–HCl–iron assay and is expected to yield better recovery of bound condensed tannins in most feed/food samples, although the recovery of bound condensed tannins can also be incomplete by the thiolysis procedure for samples such as dead outer bark of

trees (Matthews, Mila, Scalbert, & Donnelly, 1997). Solid state ^{13}C NMR can be used as a probe for measurement of bound condensed tannins. The advantages of this technique are that it is nondestructive of the material being analysed, resolves many separate cell wall components, and is capable of measuring the relative changes in these components produced by a particular treatment.

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