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ARTICLES

Interference of Condensed Tannin in Lignin Analyses of Dry Bean and Forage Crops

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Legumes with high concentrations of condensed tannin (pinto bean [*Phaseolus vulgaris* L.], sainfoin [*Onobrychis viciifolia* Scop.], and big trefoil [*Lotus uliginosus* Hoff.]), were compared to a selection of forages, with low or zero condensed tannin (smooth bromegrass [*Bromus inermis* Leyss], *Lotus japonicus* [Regel] K. Larsen, and alfalfa [*Medicago sativa* L.]), using four methods to estimate fiber or lignin. Protocols were validated by using semipurified condensed tannin polymers in adulteration assays that tested low-lignin tissue with polyphenolic-enriched samples. The effect on lignin assay methods by condensed tannin concentration was interpreted using a multivariate analysis. There was an overestimation of fiber or lignin in the presence of condensed tannin in the acid detergent fiber (ADF) and Klason lignin (KL) assays compared to that in the thioglycolic acid (TGA) and acid detergent lignin (ADL) methods. Sulfite reagents (present in TGA lignin method) or sequential acidic digests at high temperatures (ADF followed by ADL) were required to eliminate condensed tannin. The ADF (alone) and KL protocols are not recommended to screen nonwoody plants, such as forages, where condensed tannin has accumulated in the tissue.

KEYWORDS: ADF; ADL; alfalfa; condensed tannin; fiber; forage; Klason lignin; legume; *Lotus*; pinto bean; proanthocyanidin; smooth bromegrass; thioglycolic acid

INTRODUCTION

Fiber and lignin determinations are valuable diagnostic tools for breeding high-quality crops destined for either livestock feed or human consumption. In animal feed, crop quality refers to the ability of the feed to nourish livestock with little or no nutritional loss due to indigestibility (*1*). Investigators of animal feed quality reported that condensed tannins (CT, *syn.* proanthocyanidins) can interfere with the absorption of important nutritional components, such as protein and phosphorus when present in high concentration (*2*). In addition to the nutritional traits in livestock feed, crops for human consumption are required to be palatable, have a reasonable storage life, and retain high-quality cooking characteristics. Hence, it is crucial to have an accurate estimation of indigestible components, fiber and lignin, and of unpalatable ingredients such as CT, which can discolor the appearance of some foods, such as dry bean or noodles, during storage and reduce cooking quality.

The established assays Klason lignin, acid detergent fiber, and acid detergent lignin (KL, ADF, and ADL, respectively) describe plant fractions that are related to crop quality rather than delineating the individual compounds, lignin, cellulose, and hemicellulose. The gravimetric KL and ADL assays are chemically similar in terms of isolating a cellulose-free component of fiber, using a heated 72% sulfuric acid digest but differ in that ADL residues are preceded by ADF treatment (*3*).

In grain and forage research, a gravimetric lignin estimation may not be appropriate for use with these nonwoody samples. During an investigation of pinto bean (*Phaseolus vulgaris* L.), high concentrations of seed coat-associated CT were suspected of contributing to an overestimation of lignin, using a KL assay (*4*). The pinto bean phenotypes that differed in the rate of postharvest darkening and appeared to differ in lignin concentration were re-evaluated using the spectrophotometric thioglycolic acid (TGA) lignin assay. There were actually no significant differences in lignin concentration between the phenotypes. It was proposed that factors affecting the KL procedure be explored to establish what might be interfering in the measurement and to ensure that TGA-lignin was suitable. The TGA-lignin assay has been a useful alternative where other researchers suspected that a confounding plant product interfered in the lignin assays (*5*). High concentrations of phenolics from loblolly pine (*Pinus taeda* L.) needles were found to contaminate acid-insoluble lignin preparations (*5*). As an alternative method, the TGA-lignin assay was used and found to be free of contaminating phenolics in these pine needle assays.

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These foregoing reports emphasize the need to validate the methods used to estimate lignin in the presence of phenolic-rich plant material. The research reported here was designed to test established lignin protocols in a range of legumes and one grass forage, where there was a concurrent interest in CT concentration. Using these crops, TGA-lignin was compared with ADF/ADL and KL determinations, and these assay results were correlated with the presence of CT in the tissue.

MATERIALS AND METHODS

Plant Material. *Medicago sativa*L. (alfalfa, cv Beaver). Plants from a field nursery established in 1996 were transplanted into 18-cm pots and were maintained in a greenhouse since 2000, at 21/16 °C day/night temperatures under natural lighting. High pressure sodium lights (430 W, Son Agro, Phillips, Markham, ON, Canada) supplemented natural light and maintained a 16 h photoperiod October to May, with a minimum/maximum 130/350 μmol m^{-2} s^{-1}. The plants were trimmed to the basal mat after setting seed and allowed to regrow. Leaf (including petiole) and stem tissues were harvested from 2- and 4-month regrowth, bulked from plants in four different pots. Material taken at the basal level constituted a whole plant sample. Leaves from the top three nodes and stems (first four internodes) were harvested separately from whole plant samples.

*Onobrychis viciifolia*Scop (sainfoin, cv. Melrose). Plants were grown and harvested under the same conditions as those for alfalfa, except that leaf tissue was harvested from the top 10 to 12 cm of stem.

Lotus japonicus(Regel) K. Larsen and *Lotus uliginosus*Hoff. (big trefoil, also known as *Lotus pedunculatus*Cav.). Plants were grown and harvested under the same conditions as those for sainfoin. For testing in tissue adulteration assays, newly opened big trefoil leaves were collected separately from the other tissue (whole plant and leaf).

*Bromus inermis*Leyss (smooth bromegrass, cv. Signal). Plants from a field nursery, established from seed in 2004, were transplanted into 18-cm pots in October 2006 and maintained in a greenhouse under the same conditions as those for alfalfa. The plants were trimmed to stubble when inflorescence growth appeared. Vegetative leaf and stem material were harvested from the regrowth and taken down to the soil level. Younger leaf tissue (top 15−18 cm) was separated from the whole plant material.

*Phaseolus vulgaris*L. Seed coat from a bulked sample of pinto bean seed (cv. CDC Pintium, harvested in 2004 from field plots near Saskatoon, SK) was decorticated by hand and freeze-dried. Seed coat tissue was used as a control in the forage legume and bromegrass assays to represent lignified tissue known to contain both bound and extractable CT (*4*).

Tissue from the forages and bromegrass was air-dried and ground to pass through a 1 mm sieve (#18, USA-standard ASTME-11−61) and weighed to ± 0.1 mg. Unless otherwise stated, 100 to 200 mg ground tissue as dry matter (DM) (three to four replicates) was used in all assays. (*Lotus japonicus*, alfalfa, big trefoil, and sainfoin plant tissue were provided courtesy of M. Y. Gruber, and harvested bromegrass by C. Duncan, Forage Testing Program, Saskatoon Research Centre, Agriculture and Agri-Food Canada.)

Biochemical Assays. *Condensed Tannin Analysis.* The DMACA-HCl colorimetric assay (*6*) was used to screen all the plant samples. Seed coat of *Lens culinaris* L. cv. CDC Gold (CT-free lentil) was used as a matrix blank and subtracted as a background reading (A_{637}). Partially purified CT-polymer (*7*) from big trefoil was used initially to construct a standard curve for CT concentration calculations. Similarly purified CT-polymer from sainfoin was used subsequently (calculated as sainfoin-equivalents) after determining that the response of this CT polymer in DMACA reagent was similar to that of big trefoil (**Table 1**). (Both types of CT-polymers were gifts from M. Y. Gruber, Forage Research Program, Saskatoon Research Centre, Agriculture and Agri-Food Canada.)

Although the DMACA reagent is an analogue of vanillin and reacts broadly with flavan-containing compounds (**Table 1**), rather than specifically with only CT (*8*), the reaction was suitable for estimating CT concentration for comparison purposes between different genera and tissue types used in this investigation. An absolute quantification of CT, differentiating among CT, flavan-3,4-diols, or flavonols was not required, since the presence or absence of CT had been previously verified in the legumes (*9*) and is reported to be absent in temperate grasses such as bromegrass (*10*).

Table 1. Reference Standards for CT Quantification by DMACA-HCl, with a Comparison to Other Phenylpropanoids and Flavonoids

common name	source	DMACA[a]	RT[b]	UV spectral maxima[c]
(±)-catechin[d]	Sigma	+	13.5	205.1, 226.5^{sh}, 279.0
chlorogenic acid	Sigma	−	7.9	210, 290^{sh}, 325
condensed tannin[e]	in-house, AAFC	+	3.1	200.0, 264.0
(−)-epicatechin	Sigma	+	14.3	205.1, 226.5^{sh}, 276.3
isorhamnetin	Apin	tr	20.3	200, 250.3, 300^{sh}, 370.2
kaempferol	Fluka	+	20.3	202.2, 266.1., 320.0^{sh}, 366.0
naringenin chalcone	Sigma	tr	nd[f]	nd
quercetin	Sigma	tr	18.9	203.4, 253.0, 366.2
trans-cinnamic acid	Sigma	−	19.8	278.6

[a] Reaction rated as +, (tr) (≤0.01 AU), or − (see DMACA assay, in Materials and Methods). [b] Retention time, min, ± 0.05, HPLC separation (*4*). [c] nm, using UV-photodiode array; ^{sh}, shoulder. [d] (+)-Catechin and (−)-catechin (tested with purified enantiomers, Sigma) did not differ from the racemic mixture using either the DMACA assay or RT and UV-spectra. [e] Purified from big trefoil or sainfoin (not different, with respect to RT or UV-spectrum). [f] Not determined.

Lignin Determinations. (1) Thioglycolic Acid Lignin (TGA-Lignin). A modified spectrophotometric assay for small sample sizes was used (*11*) and quantified by a standard curve using a TGA-lignin standard prepared in bulk (*4*), which had been verified against a milled spruce lignin standard (gift of P. Watson, Pulp and Paper Institute of Canada, Vancouver, B.C.) (*12*). Thioglycolic acid (*syn.* 2-thiolethanoic acid or α-mercaptoacetic acid) was obtained from Sigma (Oakville, ON, Canada), and all manipulations were conducted in a fumehood.

(2) Acid Detergent Fiber (ADF) and (3) Acid Detergent Lignin (ADL) (Gravimetric) Assays. ADF/ADL assays were accomplished with a digestion apparatus and methodology according to the Ankom protocols (Ankom Technology 200/220 fiber analyzer, Macedon, NY, USA). These protocols are based on the AOAC Official Method (973.18, rev. 2000) for ADF and the subsequent ADL assay. ADL is always preceded by the ADF procedure.

(4) Acid-Insoluble Klason Lignin (KL) Assay. Gravimetric assays were performed according to a modified KL assay for small sample sizes (*13*).

Interference Assays. Preweighed sainfoin CT-polymer was added to six 100 mg (±0.1) samples of selected whole plant tissue (*L. japonicus* whole plant tissue [no CT] and pinto bean seed coat [high CT]) in order to augment the assay with a predetermined amount of CT. Control assays contained the CT-polymer alone or unadulterated (i.e., no CT-poymer) *L. japonicus* tissue, which is CT-free. Samples were processed using the protocols described previously.

To establish whether the dried CT-polymer introduced an insoluble artifact (i.e., the acidic aqueous assay conditions might prevent the polymer from dissolving), the adulterant was tested in lignin assays both as a dried augmentation to the assay or dissolved first in 100% methanol (MeOH) to ensure that it was initially in solution. In a subsequent test, equal amounts of newly opened big trefoil leaf tissue (having high CT concentration but expected to add minimal lignin) were added to the whole plant tissue of smooth bromegrass and of *L. japonicus* (tissues having no detectable CT) in triplicate assays. These adulterated samples were processed using three different lignin protocols along with the unadulterated samples.

Controls. Semipurified CT-polymer was used as a positive control for recovery from the lignin determinations. Cellulose controls (100% cotton balls, Exact, Loblaws, Brampton, ON, Canada) were used as a negative control for carry-over of this complex polysaccharide in all of the gravimetric and spectrophotometric lignin assays. An average of four crucible blanks (no sample) was subtracted in the ignition step. In the Ankom method, bag blanks were processed for each run and subtracted from the final values in that run. Final sample values were expressed as weight (mg g^{-1} DM) and were averaged from tests of at least three replicates of bulked tissue samples, each weighed to the nearest 0.1 mg. Carryover of residues in the CT-control and CT-

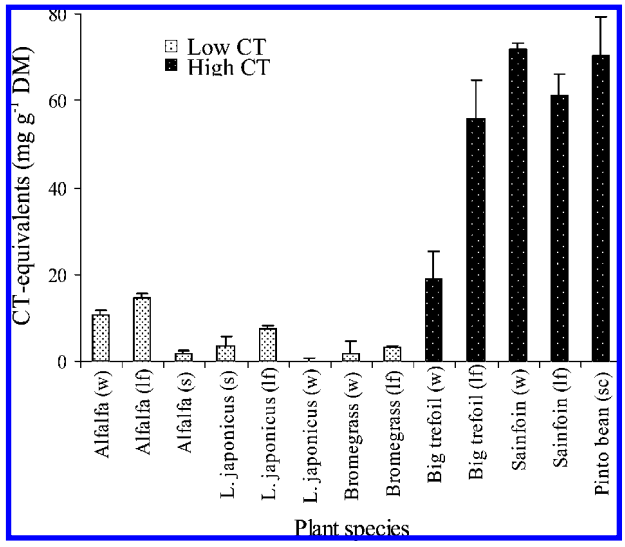

Figure 1. Condensed tannin concentration in plant species used to evaluate lignin assays. Extracts from whole plant (w), leaf (lf), or stem (s) dissections showed a significant difference in CT concentration (mg g^{-1} DM) between the designated high and low CT-concentration groups. (In the high-CT group, big trefoil whole plant contained the least amount of CT, but was grouped with sainfoin and pinto bean because of the high amount of CT in the leaf-only tissue.)

adulteration assays was calculated by dividing the final weight by the starting weight and converting to percentage.

Statistical Analysis. Significant differences in the biochemical estimates were tested using Student's *t*-test and are reported as probability (*p*) values at the 95% level. Data for individual samples were averaged from 3 or more replicates, and standard error of the means (SE) were used to determine error bars on graphs for individual plant samples. Data for means reported for each plant species grouping were from all of the replicates in the group specified, and error bars for the averaged data represent the SE over all replicates (*n*, given on the figures, where applicable). Multivariate analysis to examine the influence of the CT concentration in the tissue (high-and low-CT categories) with respect to the lignin assay method was determined by principal components analysis (PCA) based on a correlation cross-products matrix using the PC-ORD software (*14*).

RESULTS AND DISCUSSION

Comparison of Condensed Tannin Concentration in Whole Plant and in Dissected Tissues. The plant species used in the lignin quantification experiments were grouped into two classes, relative to CT concentration: (1) species with low or absent CT (alfalfa, *Lotus japonicus* and smooth bromegrass) and (2) species with high CT concentration (big trefoil, sainfoin, and pinto bean seed coat) (**Figure 1**). There was a significant difference in CT concentration between these two groups ($p \leq 0.0001$; $n = 33$), irrespective of the tissue sampled. In agreement with previous surveys (*15, 16*), we found that preparations of sainfoin and big trefoil leaf tissue produced extracts with a high concentration of CT compared to that of other forages. The seed coat extract of pinto bean also was high in CT (**Figure 1**).

Although *L. japonicus* leaf tissue is known to be deficient in CT biosynthesis (*17*), the DMACA-HCl assays signified that a DMACA-positive reaction occurred. Subsequent evaluation of these specific *Lotus* extracts by UV-spectra indicated that CT was undetectable (Marles, M. A. S., unpublished work); however, an unidentified flavonol, with a UV-spectrum similar to quercetin, was distinguished. This finding may also be used to interpret the positive reaction for CT in alfalfa and underscores the need to exercise caution when a DMACA

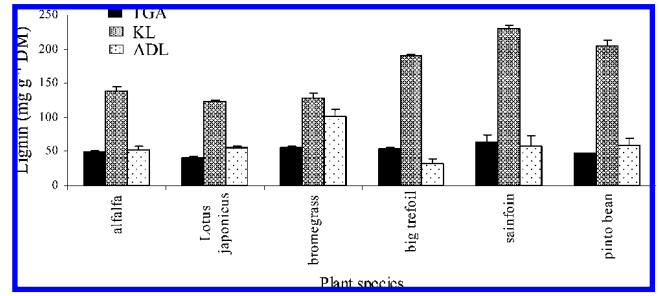

Figure 2. Comparison of lignin assay methods on whole plant tissue samples to illustrate the effect of CT on acid-insoluble residues. Lignin (mg g^{-1} DM) in tissue with no detectable CT (alfalfa, *L. japonicus* and bromegrass) was significantly different from the tissue samples with high CT concentration (big trefoil, sainfoin, and pinto bean) when KL assays were compared. Lignin concentration did not differ significantly when TGA and ADL assays were used to estimate lignin (SE, error bars).

reagent is used to quantify CT in unfractionated extracts. Concurrent research indicated that dry bean seed coat was a complex mixture of bound and extractable CT as well as other polyphenolics, which nonspecifically reacted with DMACA-HCl (*4*). Despite the lack of absolute specificity (**Table 1**), the DMACA-HCl colorimetric assay served to distinguish two categories of plants on the basis of CT concentration.

Comparison of Different Lignin Assay Methods for Screening Forages and Legumes. When KL determinations were compared with respect to CT-grouping, the results for tissue with no detectable CT (alfalfa, *L. japonicus* and bromegrass) were significantly different from the samples with high CT concentration (big trefoil, sainfoin, and pinto bean) ($p \leq 0.0001$; $n = 35$) (**Figure 2**). Theander and co-workers were satisfied that the KL method was appropriate for an estimate of digestibility, even though they reported that CT could cross-link with lignin moieties and add to the insoluble complex of cell wall debris (*18, 19*). However, lignin concentration did not differ appreciably when TGA assays of whole plant samples were used as an estimate, irrespective of CT concentration in the tissues (**Figure 2**). Compared to TGA-lignin assays, KL determinations were notably sensitive to CT concentration.

In ADF assays of whole plant samples, the whole-plant data were also not significantly different between the CT concentration groupings ($p = 0.1804$). However, when young immature leaf-only tissue was tested, the ADF values from the low-CT group were marginally different compared to that of the high-CT group ($p = 0.0739$). This difference may not necessarily be reflective of CT concentration though because ADF is an estimate of dietary fiber that includes cellulose and hemicellulose. In discussing fiber and lignin estimates generated from carbohydrate-free, sulfuric acid-insoluble residues in green crops, the caveat has been offered that there can be an overestimation related to unspecified Maillard products, phenolic-related esters, and polyphenolic carry-over (*20*−*22*). When evaluating lignin in protein-rich forages, CT-rich legume samples were cited as particularly troublesome.

Although an actual measure of assay sensitivity to CT or specific identification of CT was not reported in the foregoing articles, the presence of CT was nonetheless suggested to be correlated with increased ADF and ADL estimates (*21, 23, 24*). Although it was evident in our study that the ADL method did not completely eliminate CT, ADL estimates did not appear to be significantly compromised by CT concentration. By comparison to ADL, the TGA-assay removed interfering CT and was a highly reproducible procedure (**Figures 2** and **3**).

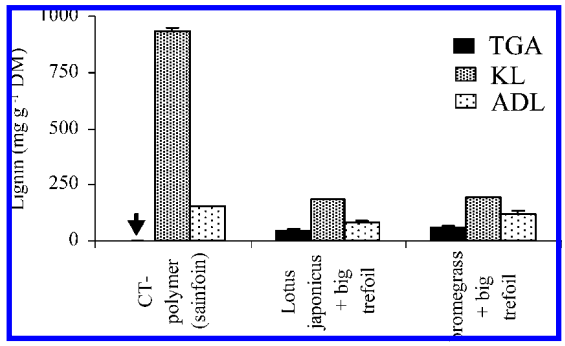

Figure 3. Control (sainfoin CT-polymer alone) and adulterated lignin analyses. The CT-polymer represented a significant pseudo-lignin moiety in KL and ADL but was undetectable in the TGA-lignin assay (↓). The adulteration assays, using a constant mass of developing leaf tissue from big trefoil (as a source of CT), display similar confounding effects due to CT in the big trefoil tissue in assays with different types of CT-free tissue (*L. japonicus* and bromegrass). (Error bars, standard error of the mean).

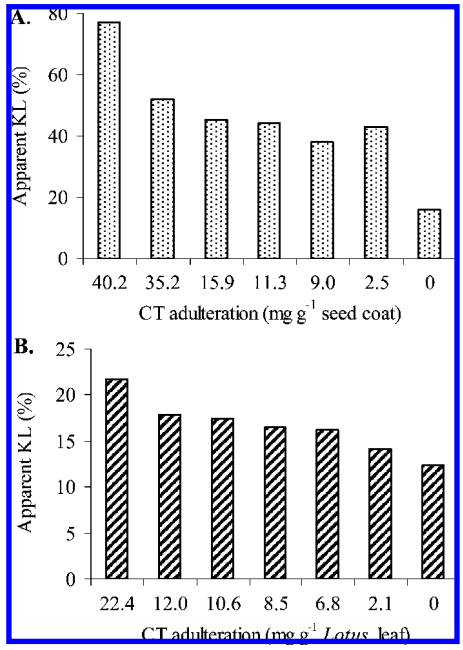

Figure 4. Effect of adulterating KL assays with sainfoin CT-polymer in (**A**) pinto bean seed coat tissue and (**B**) *Lotus japonicus* leaf tissue. One hundred milligram aliquots of two different tissue types were mixed individually with increasing amounts of the sainfoin CT-polymer. The % apparent carry over was calculated by (final weight ÷ starting weight) × 100.

Efficacy of the TGA-Lignin Protocol Underscores Advantages of Thiol-Based Reagents. The KL determination of leaf tissue of *L. japonicus* (where CT is absent) adulterated with big trefoil (which contains high concentrations of CT) (**Figure 3**), together with the data from tissue adulterated with CT polymer (**Figure 4**), produced results consistent with the hypothesis that certain types of acid digest protocols such as the KL method overestimates lignin in the presence of CT. These results invite a closer examination of assay protocols. In the elimination of CT during TGA-lignin isolation, the salient reagent may be the inclusion of a sulfite ingredient in the initial acidic digest. Sulfite reagents such as thioglycolic acid contribute to the solubilization of hemicellulose and other complex carbohydrates, including hydroxyquinones, and this reaction was the reason for including it in assay protocols of woody, and

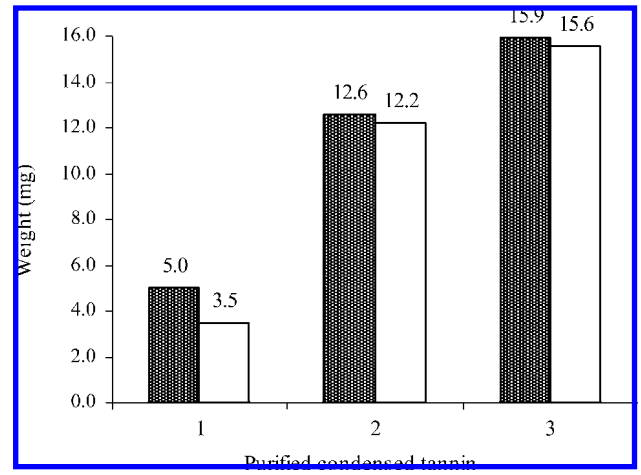

Figure 5. Klason lignin assay with sainfoin CT-polymer alone, showing recovery (mg) in the final residues. Three different starting sample masses were used (5.0, 12.6, 15.9 mg, filled bars) in the assay. A minimum of 70% (sample #1) of the polymer was carried over to contribute to the final gravimetric measurement (open bars). The % apparent carry over was calculated by (final weight ÷ starting weight) × 100. Over 90% of the CT-polymer was recovered in CT samples 2 and 3.

later, herbaceous plant material (*27, 28*). The effectiveness of a sulfite reagent in lignin assays has been described in a variety of legumes including clover (*Trifolium pretense* L.), sericea (*Lespedeza cuneata* [Dum. Cours.] G. Don.), and several vetch species (*24*). These researchers showed that the detergent methods overestimated ADF, NDF, and ADL in the high CT-containing samples. Their results were positively correlated with the inclusion versus the exclusion of sodium sulfite in the acid−based residue extractions. The investigators also speculated that polyphenol oxidase conversion formed insoluble condensation products (with protein) and that such complexes were eliminated with a sulfite reagent.

Confirmation of Condensed Tannin As a Confounding Factor in Lignin Determinations. Sensitivity of fiber and lignin assays to CT interference was first measured by processing sainfoin CT-polymer alone, as a control (**Figure 3**, sainfoin CT-polymer). In the TGA-lignin assay (with CT-polymer alone), there was no detectable carryover (arrow, **Figure 3**), although a considerable CT-residue was recovered in the gravimetric KL assay and to a certain extent in the ADL assay (**Figure 3**). The KL method showed the most carryover in the control assay, retaining at least 70% of the original amount of the starting weight of the CT polymer, even when the CT was first dissolved in MeOH (**Figure 3**, control assays). The ADL residue, which was similar to the TGA-lignin estimate of lignin, was represented by 15.6% carryover of CT-polymer (**Figure 3**, control polymer).

Assays of plant samples augmented with known amounts of CT-polymer may introduce an artifact since the adulterant is not biologically the same as the naturally occurring material. We addressed this issue by adulterating samples of different plant species (*L. japonicus* and bromegrass) having a naturally low CT-concentration, with a constant mass of big trefoil leaves, an unprocessed biological source of high CT (**Figure 3**). The results of these experiments showed that CT-rich tissue could affect the gravimetric assays, just as was determined in the control assay samples with the CT-polymer. Assay results for unadulterated tissue as a comparison are shown in **Figure 2**.

The effect of CT on a heterogeneous polymer such as lignin can be difficult to evaluate accurately in lignin digests. Hence, further documentation of the effects of CT-polymer on KL

estimates was pursued, following the observation that over 90% of the CT could persist in the assay (**Figure 4**). Three different masses of CT-polymer were used (5.0, 12.6, and 15.9 mg), with no additional plant tissue and subjected to a KL assay (**Figure 5**). A minimum of 70% of the polymer was carried over to contribute to the final gravimetric measurement (5.0 mg sample, **Figure 5**). Over 90% of the CT-polymer was recovered in samples with higher starting masses (12.6 and 15.9 mg). The lower recovery (70%) in the smaller starting weight of sample #1 (**Figure 5**) was indicative of the precision of the assay, rather than a function of assay insensitivity. This lower limitation in assay precision was originally determined in a preliminary KL assay with CT-polymer alone. CT-polymer masses below 10 mg yielded results comparable to those of **Figure 5**; however, the variation was much greater, thereby emphasizing the lower limit of reliable detection.

When the CT-polymer was digested in sulfuric acid, starting as dry matter like the plant samples, the carryover of CT-related residues was at least 96%. This outcome implied that a predigest of hot MeOH, similar to the TGA-lignin method, may be important in initiating KL assays with nonwoody plant material. However, a predigest treatment may still not address problems with the carryover of a CT-related residue because cross-linked (bound) or unextractable CT that has been converted by polyphenol oxidase to form quinones behaves as an inert moiety in hot MeOH (*12, 24*) and would still contaminate the residue.

The results of the adulterated KL assay of pinto bean seed coat (**Figure 4A**) were similar to those of adulterated *L. japonicus* leaf tissue (**Figure 4B**). As the amount of CT-adulterant increased, so did the apparent KL value, even though the actual amount of plant tissue remained the same. It was evident that CT was influencing the outcome of the KL assays irrespective of the native tissues, regardless of the absolute KL concentration range in the pinto bean seed coat (**Figure 4A**) or in the leaf samples of *L. japonicus* (**Figure 4B**).

Polyphenolics, flavonoids in particular, are widely occurring secondary metabolites whose behavior as a general group *in vivo* is by no means uniform. These compounds can assume a variety of solubilities and cross-linkages with cellular components, depending on the substituents on the phenolic molecule. In addition, biotransformation (e.g., oxidation to quinones or polymerization), auto-oxidation, and nonenzymatic cross-linking to cell wall material, carbohydrates, or protein can promote unextractable residues (*25, 26*). The influence of CT on the apparent lignin concentration in a gravimetric assay is therefore not surprising.

Modeling the Interaction of CT Concentration and Lignin Assay Methods. A multivariate PCA analysis provided a perspective of the relative influence of CT on the four lignin assays within the context of an ordination of the five components. The analysis produced a separation of the high- (▲) and low-CT (Δ) groups along three axes (accounting for 42.1%, 31.6%, and 17.2% of the variance, respectively) (**Figure 6**). The scatter of individuals within each group on the ordination indicated the degree of variability among the individuals in a two-dimensional space with respect to the five types of assays (CT, KL, TGA, ADF, and ADL) (**Figure 6**). The data for CT and KL influenced the clustering most strongly and together were highly correlated ($r^2 = 0.843$ and 0.812, respectively) (ordination model, dotted lines, with corresponding lines labeled by test [CT, KL, etc.]). This indicated that KL was significantly affected by CT concentration because the positions of KL and CT in the ordination demonstrated that these traits associated more strongly with each other than with the other lignin assays.

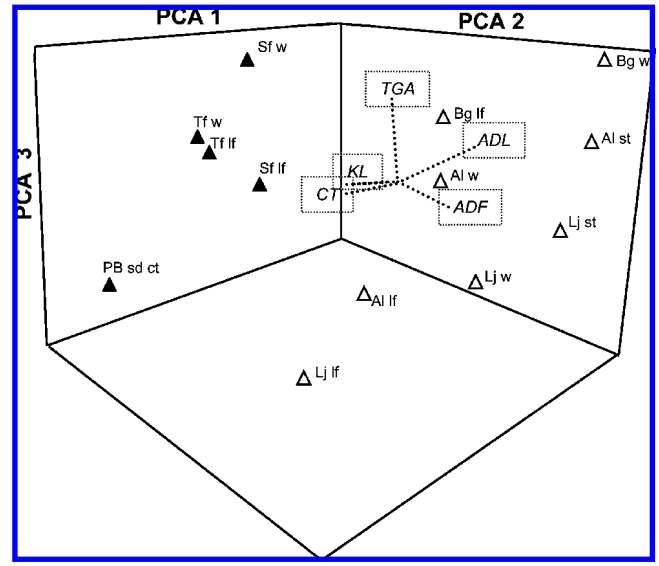

Figure 6. Principal components analysis of the lignin assay methods (KL, TGA, ADL, and ADF) that influence the clustering of the tissues according to CT-grouping. A three-dimensional model of the 3 significant axes, where the distance among objects approximate their Euclidean distances. Ordination (···) is overlaid in the 3-D plot. Legend: high-CT (▲), low-CT (Δ). Tissue: lf, leaf; st, stem; w, whole plant; sd ct, seed coat. Species: Al, alfalfa; Bg, bromegrass; Lj, *Lotus japonicus*; PB, pinto bean; Sf, sainfoin.

Conversely, ADF and ADL were associated with each other but did not have a significant effect on the separation along the first axis (**Figure 6**). However, ADL was significantly correlated with separation of the samples on the second axis ($r^2 = 0.816$). From the perspective of a three-dimensional space, ADF was weakly associated ($r^2 = 0.616$) with the second principal component (**Figure 6**). Considering that ADF is more than just a measure of acid-insoluble lignin, its weaker correlation with the clustering of samples of either high or low CT concentration was to be expected.

The TGA data in the matrix was responsible for further separation of CT groups in the PCA ordination on the third axis (17.2% of the variance). In the ordination model, TGA data influenced the third axis (component) (dotted line labeled TGA, **Figure 6**). Although the third axis was significant, the correlation with CT clustering on the third axis was not strong (TGA, $r^2 = 0.446$; CT, $r^2 = 0.105$), indicating that TGA values were not influenced by the CT data. Therefore, the TGA-lignin data emerged according to this model as free of interference by CT.

In legume cultivar development, it is essential that assay methods accurately evaluate the parameter in question in order for plant breeders to address complex quality traits. In this way, researchers have a better chance at teasing out the appropriate phenotypes in a segregating population. To this end, it is evident that determination of CT and lignin concentrations would be more useful when they can be tested independently of each other.

ABBREVIATIONS USED

ADF, acid detergent fiber; ADL, acid detergent lignin; aq., aqueous; CT, condensed tannin; DMACA, 4-(dimethyl-amino) cinnamaldehyde; DM, dry matter; KL, Klason lignin; MeOH, methanol; PCA, principal components analysis; SE, standard error of the mean; TGA, thioglycolic acid.

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