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Acetone Enhances the Direct Analysis of Procyanidin- and Prodelphinidin-Based Condensed Tannins in *Lotus* Species by the Butanol–HCl–Iron Assay

John H. Grabber,*[,†] Wayne E. Zeller,[†] and Irene Mueller-Harvey[‡]

[†]U.S. Dairy Forage Research Center, Agricultural Research Service, U.S. Department of Agriculture, 1925 Linden Drive West, Madison, Wisconsin 53706, United States

[‡]Chemistry and Biochemistry Laboratory, Food Production and Quality Division, School of Agriculture, Policy and Development, University of Reading, P.O. Box 236, 1 Earley Gate, Reading RG6 6AT, U.K.

Supporting Information

ABSTRACT: The butanol–HCl spectrophotometric assay is widely used for quantifying extractable and insoluble condensed tannins (CT, syn. proanthocyanidins) in foods, feeds, and foliage of herbaceous and woody plants, but the method underestimates total CT content when applied directly to plant material. To improve CT quantitation, we tested various cosolvents with butanol–HCl and found that acetone increased anthocyanidin yields from two forage *Lotus* species having contrasting procyanidin and prodelphinidin compositions. A butanol–HCl–iron assay run with 50% (v/v) acetone gave linear responses with *Lotus* CT standards and increased estimates of total CT in *Lotus* herbage and leaves by up to 3.2-fold over the conventional method run without acetone. The use of thiolysis to determine the purity of CT standards further improved quantitation. Gel-state ^{13}C and $^{1}H-^{13}C$ HSQC NMR spectra of insoluble residues collected after butanol–HCl assays revealed that acetone increased anthocyanidin yields by facilitating complete solubilization of CT from tissue.

KEYWORDS: *Condensed tannins, proanthocyanidins, HCl–butanol assay, gel-state nuclear magnetic resonance, thiolysis, Lotus corniculatus, Lotus uliginosus*

INTRODUCTION

The butanol–HCl assay is among the most specific and commonly used spectrophotometric methods for quantifying condensed tannins (CT) in forages, foods, feeds, and foliage of herbaceous and woody plants.[1−3] The method is based on the acid-catalyzed oxidative depolymerization of extension units in CT to produce colored anthocyanidins. Originally developed for the analysis of CT in plant extracts, the method was subsequently adapted to measure insoluble CT in solvent- or detergent-extracted residues and total CT by direct analysis of plant material.[4−8] Studies with the butanol–HCl method, later confirmed by thiolysis and other techniques, revealed that a substantial portion of CT in many plants exists in forms that cannot be extracted by aqueous organic solvents or detergents.[5,9,10]

Although simpler and more versatile than many analytical methods for quantifying CT, the butanol–HCl assay suffers from several shortcomings. First, anthocyanidin color yields are influenced by the type of CT being assayed and by side reactions during CT conversion into anthocyanidins.[1,8] This limitation is best overcome by using CT standards from the plant materials under study.[1,2,11] Second, anthocyanidin color yields are greatly influenced by the conditions under which the butanol–HCl assays are performed. In most applications, CT-containing samples are heated at 95 °C in *n*-butanol containing 5% of concentrated HCl.[1,2] Water added with HCl and plant extracts greatly affects color yields and a water content around 7% is often, but not always, optimal for maximizing the sensitivity and reproducibility of the assay.[8,12] Aqueous solutions of acetone or other organic solvents added with CT extracts can reduce anthocyanidin yields,[13] but this response might be due to water rather than the organic solvent per se. Iron(III) is often added as an oxidant to increase color yields from extracted CT, but reports differ on whether iron enhances or detracts from the reproducibility of the assay.[5,8,12] Recommended heating times vary from 10 min to 2 h and this is likely related to the characteristics of CT under study and the makeup of the butanol–HCl reagent.[13] Thus, while rarely done, conditions for the assay should be verified for each type of CT undergoing analysis, and both standards and CT-containing samples must be run concurrently in identically formulated butanol–HCl reagents.

A third often overlooked issue is that the near universally adopted butanol-based reaction medium developed over 50 years ago for isolated CT[7] has been applied essentially without verification for the direct analysis of insoluble CT or total CT in plant material. Studies by Makkar et al.[14] revealed the shortcomings of this approach by demonstrating that typical butanol–HCl assay conditions incompletely depolymerized CT contained in detergent-extracted residues or intact plant tissue. In this paper, we describe studies identifying acetone as a superior cosolvent in the butanol–HCl–iron medium for increasing CT solubilization and anthocyanidin color yields

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from tissue and for improving CT quantitation when used in conjunction with CT standards of known purity. The studies were conducted with birdsfoot trefoil (BFT, *Lotus corniculatus* L.) and big trefoil (BT, *Lotus uliginosus* Schkuhr), tanniferous forage legumes with contrasting procyanidin (PC) and prodelphinidin (PD) compositions that were chosen in part to represent the most common forms of CT found in plant materials.

MATERIALS AND METHODS

Tissues Used for CT Isolation and Analysis. In 2006, freeze-dried whole-plant herbage of 40-d-old summer regrowth of BFT, BT, and alfalfa (*Medicago sativa* L.) was ground with a cyclone mill to pass a 1 mm screen. In 2009, 45-d-old summer regrowth of BFT and BT was freeze-dried and then shaken to recover leaves, which were ground with a cyclone mill to pass a 0.5 mm screen.

Herbage CT Isolated by Batch Sephadex LH-20. Herbage CT extracted by acetone−water was isolated by a batch procedure using Sephadex LH-20 resin prepared in 80% ethanol.[15] Briefly, BFT and BT herbage (1.5 g) was sonicated (15 min) and pelleted (3,000*g*, 10 min) three times in 30 mL of 3:1 (v/v) acetone/water containing 1% (v/v) acetic acid. After filtering, the combined supernatants were extracted three times with an equal volume of diethyl ether, and the aqueous phase was partially concentrated in vacuo by rotary evaporation (<40 °C) to remove residual organic solvents. The aqueous phase was stirred for 5 min in a beaker with a one-half volume of Sephadex LH-20 resin, and the resulting slurry was decanted onto a fritted glass filter and washed with 95% ethanol until the filtrate was colorless. The resin was then washed with 3:1 acetone/water until brownish CT eluted. After in vacuo rotary evaporation (<40 °C) to remove acetone, isolated herbage CT was lyophilized and dissolved in methanol (5 mg mL^{-1}) for use in butanol−HCl assays.

Leaf CT Isolated by Column Fractionation with Toyopearl HW-55F. Leaf CT fractions were prepared by extracting BFT and BT leaves (20 g) with 7:3 acetone−water (200 mL) for 40 min. Extracts were filtered and extracted with dichloromethane (200 mL), and the aqueous phase was collected and concentrated in vacuo by rotary evaporation (<40 °C) to remove residual organic solvents and then freeze-dried, yielding 4.8 and 5.4 g of crude BFT and BT extracts, respectively. Each crude extract (1 g) was dissolved in a minimal volume of 3:7 methanol/water and applied to a column (15 cm height × 3 cm diameter) of Toyopearl HW-55F (TOSOH Bioscience GmbH, Stuttgart, Germany) prepared with water. After washing with 3:7 methanol/water (200 mL) and 7:3 methanol/water (200 mL), the column was eluted with a series of acetone/water mixtures (3:7, 200 mL; 1:1, 200 mL; and 7:3, 200 mL). All acetone/water fractions were recovered and dried in vacuo by rotary evaporation (<40 °C), followed by freeze-drying. Fractions were dissolved in methanol (4 mg mL^{-1}) and analyzed in triplicate by thiolysis with benzyl mercaptan at 40 °C for 30 min for composition and purity.[16] Flavanols and their thiol derivatives were detected at 280 nm and peak areas integrated using previously published response factors.[16] Benzyl mercaptan releases the monomeric catechin or epicatechin flavanols (terminal units) and their corresponding thiol derivatives (extension units) from PC polymers; similarly, gallocatechin or epigallocatechin (terminal units) and their corresponding thiol derivatives (extension units) are released from PD polymers. The CT content (or purity) of the fractions was obtained from the sum of all flavanols and thiol derivatives (mass basis) and are reported as g CT/100 g fraction. The fractions of highest purity from BFT (eluted with 7:3 acetone/water) and BT (eluted with 1:1 acetone/water) were used as CT standards for NMR analyses and dissolved in methanol (5 mg mL^{-1}) for use in butanol−HCl assays.

General Methods for Butanol−HCl Assays. Butanol−HCl reagents were prepared fresh daily on a per 100 mL basis by first stirring 40 mg of ammonium iron(III) sulfate dodecahydrate in 3.3 mL of water and 5.0 mL of 12 M HCl for 30 min and then adding 92 mL of *n*-butanol. Cosolvents, if used, were added last with a concomitant reduction in butanol volume. Assays of herbage and leaf tissues and of isolated CT were carried out concurrently with 15 mL of butanol−HCl reagent in 25 mL capacity thick-walled glass tubes sealed with Teflon-lined screw caps. Tubes were heated as described below in an aluminum block placed within a shielded fume hood. Samples were briefly vortexed every 15 min during heating and then air-cooled for about 45 min to room temperature. Reaction mixtures with tissue were decanted into 2 mL conical polypropylene-copolymer microcentrifuge tubes, sealed with screw caps, and centrifuged for 2 min at 10 000*g*. After centrifugation, clarified supernatants were scanned with a spectrophotometer from 400 to 600 nm, and the maximal absorption of the anthocyanidin peak was recorded. Butanol−HCl reagents were used as blanks and as a diluent to keep maximal absorbance readings of anthocyanidin peaks from tissues below 1.5 units. Reactions with isolated CT were scanned in a similar manner, but without centrifugation or dilution. To avoid evaporative losses of solvents, reagents and reaction mixtures were kept in sealed vessels except for brief intervals during pipetting and scanning of each sample. Experimental treatments were replicated at least twice by carrying out assays on separate days with freshly prepared butanol−HCl reagents.

Solvent and Iron Effects on Anthocyanidin Production. Assays were carried out with 0.25 mg of isolated CT or 30 mg of tissue prepared from BFT and BT herbage and leaves. To screen cosolvents for enhancing anthocyanidin color yields, leaves were heated at 95 °C for 1 h in butanol−HCl−iron reagent formulated with 10% (v/v) of various polar aprotic, polar protic, and nonpolar solvents (see Table 1). The response of anthocyanidin production to butanol−HCl−iron reagents formulated with 0, 20, 40, or 60% (v/v) acetone were then determined by heating herbage and isolated herbage CT at 70 °C for 0.5, 1, 2, or 3 h and by heating leaves and isolated leaf CT standards at 70 °C for 1, 2, 3, or 4 h. Finally, the effects of iron in the assay were determined by heating herbage and isolated herbage CT at 70 °C for 2.5 h in butanol−HCl reagents formulated with 0, 0.2, 0.4, or 0.6 mg mL^{-1} of ammonium iron(III) sulfate dodecahydrate and 0, 20, 40, or 60% acetone. Absorption data from experiments examining solvent and iron effects on anthocyanidin production are expressed as a specific absorption coefficient (SAC), which is the absorbance per unit path length (cm) per unit of mass concentration (g of tissue or isolated CT per liter of reagent).

Table 1. Specific Absorption Coefficient (SAC) of Anthocyanidins Produced from Condensed Tannins (CT) in Birdsfoot Trefoil (BFT) and Big Trefoil (BT) Leaves Heated at 95 °C for 1 h in Butanol−HCl−Iron Reagent Amended with 10% of Various Cosolvents (N = 3)

	SAC	
cosolvent	BFT	BT
butanol (control)	0.74 g[a]	0.65 f
acetone	1.05 ab	1.61 a
butanone	0.94 bcd	1.16 bc
3-pentanone	0.88 cde	0.96 de
cyclohexanone	1.05 a	1.65 a
methanol	0.78 fg	0.91 e
ethanol	0.84 efg	0.94 e
ethylene glycol	0.76 fg	0.71 f
tetraethylene glycol	0.86 def	0.71 f
ethylene glycol dimethyl ether	1.05 a	1.07 cd
di(ethylene glycol) monoethyl ether	0.91 cde	0.93 e
cyclohexane	0.53 h	0.32 g
tetrahydrofuran	1.05 a	1.24 b
dioxane	0.97 abc	1.16 bc
phenol	0.58 h	0.30 g
standard error of the mean	0.057	0.083

[a]In each column, least square means followed by unlike letters differ at P = 0.05.

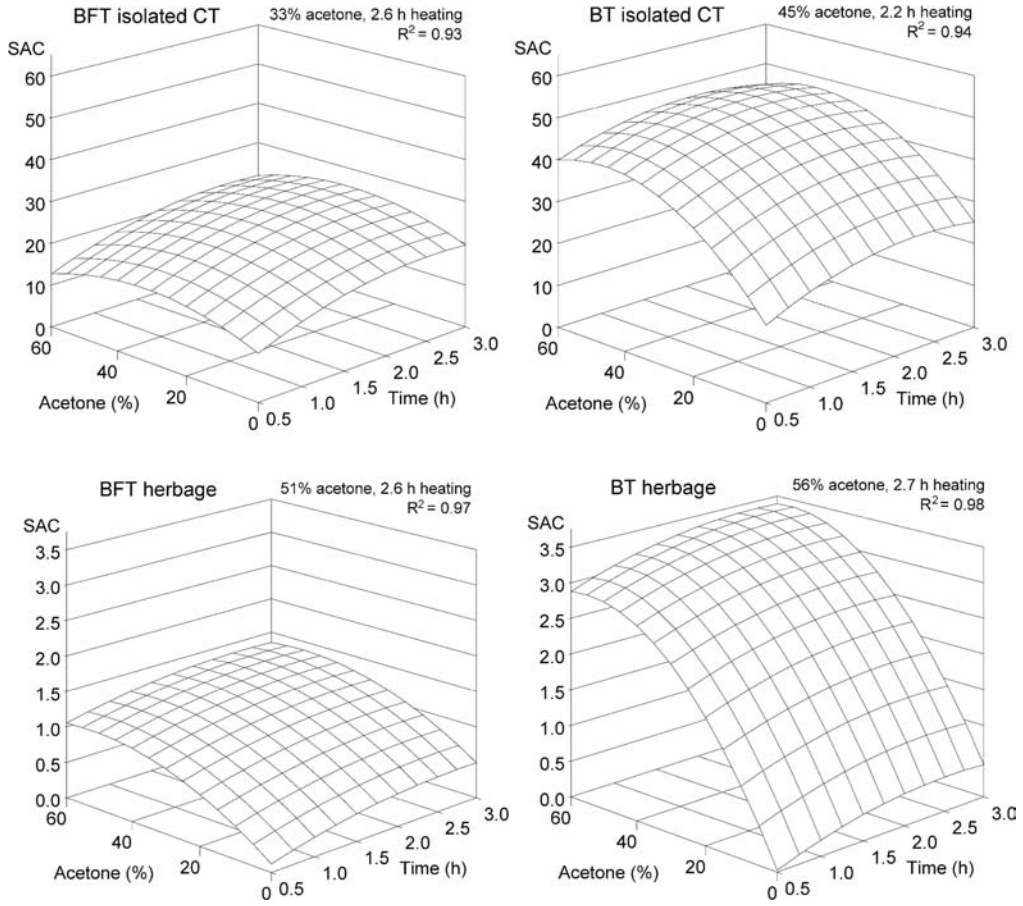

Figure 1. SAC of anthocyanidins produced from isolated herbage CT and herbage tissue of BFT and BT as influenced by the concentration of acetone in the butanol–HCl–iron reagent and heating time at 70 °C (N = 2). Response surface estimates of acetone concentrations and heating times for maximal SAC are shown.

Quantifying Total CT with the Conventional vs the Modified Assay. Isolated leaf CT standards (0.1, 0.2, 0.3, 0.4, and 0.5 mg) and herbage or leaf tissues (30 mg) were heated at 95 °C for 2.5 h with 15 mL of a conventional butanol–HCl–iron reagent and at 70 °C for 2.5 h with 15 mL of a modified acetone–butanol–HCl–iron reagent. The conventional reagent was prepared on a per 100 mL basis with 40 mg of ammonium iron(III) sulfate, 3.3 mL of water, 5.0 mL of 12 M HCl, and 92 mL of *n*-butanol. Optimal reaction time with the conventional reagent was determined by heating herbage and isolated herbage CT at 95 °C for 0.5, 1, 2, or 3 h. The modified reagent was prepared with 40 mg of ammonium iron(III) sulfate, 3.3 mL of water, 5.0 mL of 12 M HCl, 42 mL of *n*-butanol, and 50 mL of acetone. Appropriate conditions for the modified assay were ascertained from experiments described in the previous section. After heating, solutions were scanned as described above, using the conventional or the modified reagents as blanks and as diluents to keep absorbance readings below 1.5 units. Finally, contributions of nonanthocyanidin pigments to BFT and BT tissue spectra were corrected by subtracting the spectra of alfalfa herbage heated in butanol–HCl reagents. Alfalfa is morphologically and chemically comparable to BFT and BT but lacks CT.

Gel NMR Analysis of CT in Leaf Tissue and Assay Residues. For this experiment, assay residues were prepared by weighing BT leaves (30 mg) into two sets of 12 thick-walled glass tubes. One set was assayed with conventional reagent and the other with modified reagent as described in the previous section. After cooling, the contents of the tubes were decanted into 50 mL polypropylene-copolymer tubes, sealed, and centrifuged (20 000*g*) at 10 °C for 30 min. Pellets from each assay were suspended and pooled into a single tube for centrifugation, using reagent mixtures prepared without iron. After being suspended and pelleted three times in 30 mL of iron-free reagent, insoluble residues were dried in vacuo and weighed. Assays were replicated three times on separate days with freshly prepared reagents. The recovery of pooled residues from the 360 mg of BT leaves assayed in each set averaged 112 ± 10.9 mg (31% yield) for the conventional reagent and 137 ± 5.5 mg (38% yield) for the modified reagent prepared with acetone.

Methods used for the preparation and analysis of CT in leaf tissue and assay residues by gel NMR were adapted from procedures developed for the analysis of lignin and structural polysaccharides in plant cell walls.[17,18] Leaves and assay residues from BT were ball-milled at 30 Hz for 3 × 30 min runs (30 min intervals between runs) using a Retsch MM301 mixer mill equipped with dual 10 mL ZrO_2 jars and a single 10 mm ZrO_2 ball bearing per jar. Ball-milled leaves (52.0 mg) and residues from conventional and modified assays (50.2 mg each) were evenly distributed along the length of horizontally positioned 5 mm NMR tubes, wetted with DMSO-d_6 (400 μL) and pyridine-d_5 (100 μL), and then mixed and sonicated until a homogeneous gel was formed (usually <1 h). The leaf CT standard from BT (12.0 mg) was prepared for NMR in a similar manner, but sonication was not required for dissolution in 4:1 DMSO-d_6/pyridine-d_5.

^{13}C NMR and ^{1}H–^{13}C correlation 2D NMR (HSQC) spectra were recorded at 27 °C on a BrukerBiospin DMX-500 (^{1}H 500.13 MHz, ^{13}C 125.76 MHz) instrument equipped with TopSpin 2.1 software and a cryogenically cooled 5-mm TXI ^{1}H/^{13}C/^{15}N gradient probe in inverse geometry. Spectral resonances were referenced to the residual signals of DMSO-d_6 (2.49 ppm for ^{1}H and 39.5 ppm for ^{13}C spectra). ^{13}C NMR spectra were obtained using 50K scans (acquisition time 45 h 3 min each) for ball-milled leaves and assay residues and 14.3K scans (acquisition time 13 h) for the leaf CT standard. For ^{1}H–^{13}C HSQC experiments, spectra were obtained using 2400 scans (acquisition time 116 h 47 min each) for ball-milled leaf tissue and assay residues and 64

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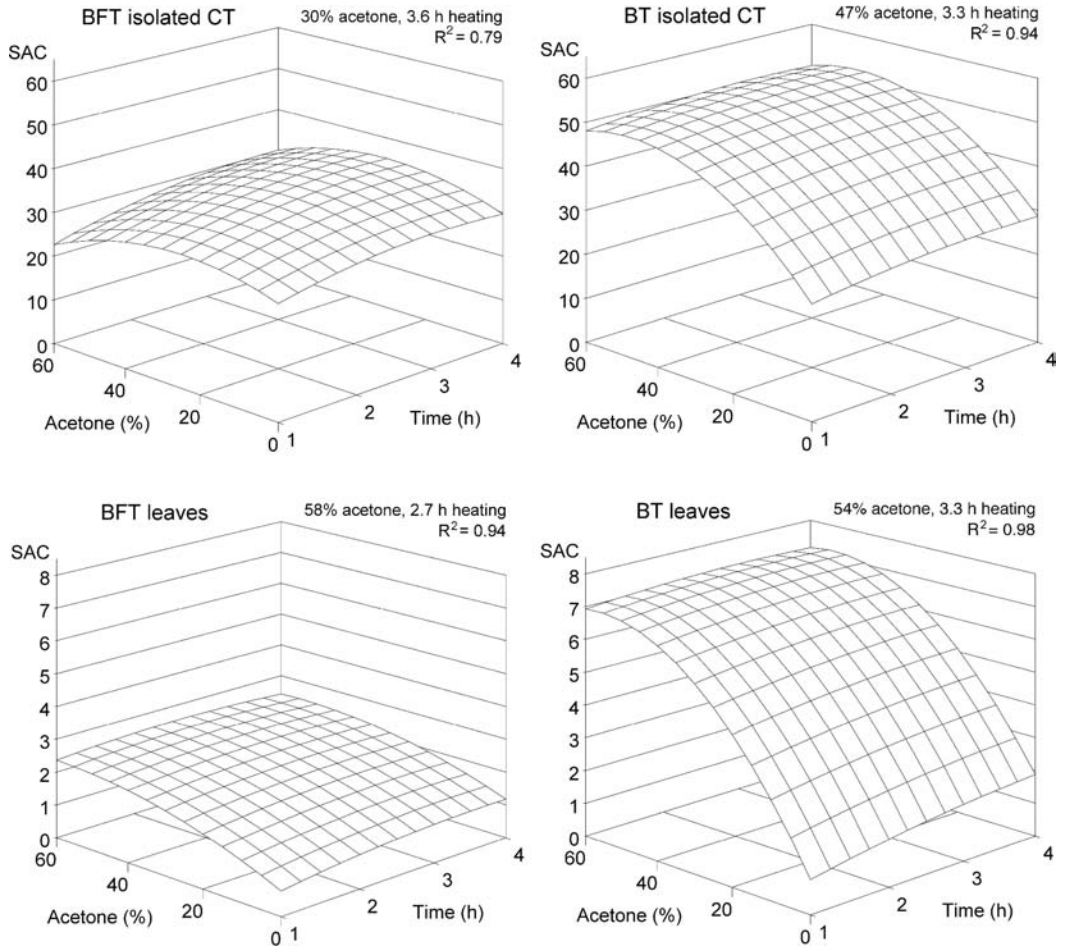

Figure 2. SAC of anthocyanidins produced from isolated leaf CT and leaf tissue of BFT and BT as influenced by the concentration of acetone in the butanol–HCl–iron reagent and heating time at 70 °C (N = 3). Response surface estimates of acetone concentrations and heating times for maximal SAC are shown.

scans (acquisition time 3 h 7 min) for the leaf CT standard. To assess detection limits in assay residues, 12.5 mg of isolated leaf CT in 500 μL of DMSO-d_6/pyridine-d_5 (4:1) was serially diluted down to $^1/_{100}$ of the initial concentration and subjected to ^{1}H–^{13}C HSQC NMR experiments using 2400 scans.

Statistical Analysis. Data were analyzed by the MIXED, RSREG, and REG procedures (SAS Institute, Cary, NC). Differences among treatment means were tested by the PDIFF procedure if F-tests were significant at P = 0.05 (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Cosolvent and Iron Effects on Anthocyanidin Production. Butanol–HCl assays are routinely carried out at 95 °C, but reported heating times range from 10 min to 2 h.[13] A median heating period of 1 h was, therefore, selected for the cosolvent screening experiment. Among the cosolvents added at a 10% level in the assay, acetone and cyclohexanone provided the greatest accentuation in anthocyanidin color production from BFT and especially BT leaves (Table 1). Butanone and 3-pentanone proved less effective, suggesting that the mere presence of a ketone moiety did not fully account for increased color formation in the assay. Aqueous acetone is commonly used to extract CT from plant materials,[1−3,13] but to our knowledge acetone, cyclohexanone, or other ketones have not been specifically evaluated as cosolvents for improving anthocyanidin yields in the butanol–HCl assay. Methanol and ethanol, which are also commonly used as extractants for CT,[1−3,13] had little or no effect on color yields from leaves, nor did other polar protic cosolvents [ethylene glycol, tetraethylene glycol, and di(ethylene glycol) monoethyl ether]. For BFT leaves, color formation with polar aprotic cosolvents (ethylene glycol dimethyl ether, tetrahydrofuran, and dioxane) equaled that with acetone and cyclohexanone, but these particular cosolvents proved to be less effective for BT leaves. The addition of nonpolar aprotic or relatively nonpolar protic cosolvents (cyclohexane or phenol) to butanol–HCl depressed anthocyanidin color yields from leaves. Other studies with polyethylene glycol, phenol–acetic acid–water (2:1:1), and dimethyl sulfoxide indicated that they were incompatible or not effective for boosting anthocyanidin production from tissue heated in butanol–HCl (data not shown). Although both acetone and cyclohexanone initially appeared equally promising as cosolvents for the analysis of total CT, we initiated in depth studies with the former because aqueous acetone is commonly used for the isolation and analysis of extractable CT by the butanol–HCl assay.[1−3,13] Thus, this approach could potentially permit the analysis of both total and extractable CT in a similar butanol–HCl matrix containing acetone and water.

The response of anthocyanidin color yields to acetone concentration was determined by heating isolated CT, herbage, and leaves for up to 4 h in butanol–HCl–iron reagents prepared with 0–60% acetone and 7% total water (includes water contributed by HCl). Due to the high volatility of

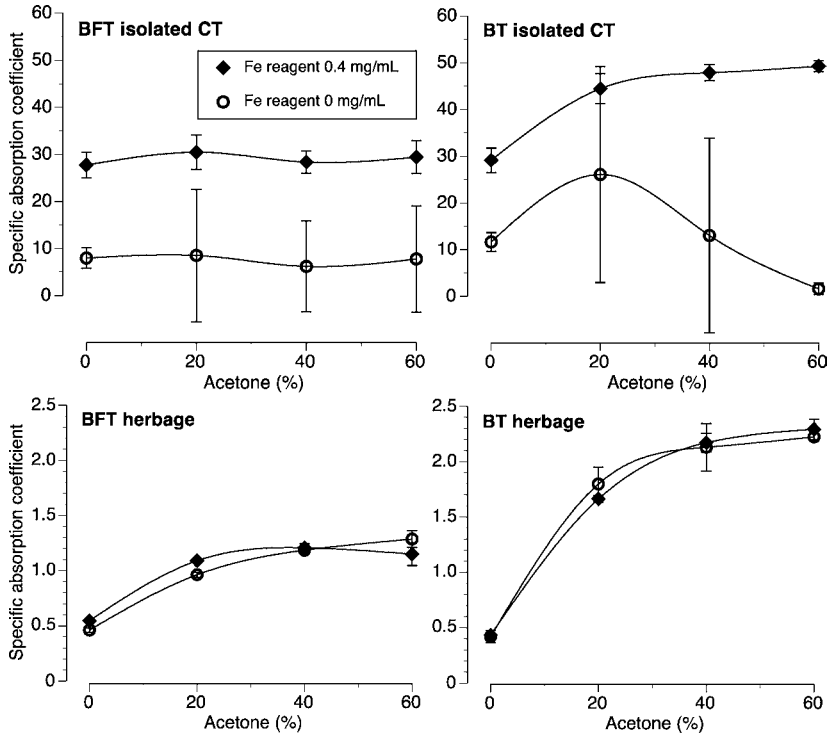

Figure 3. Anthocyanidin color yields from isolated herbage CT and herbage tissue of BFT and BT as influenced by iron and acetone concentrations in butanol−HCl−iron reagent heated at 70 °C for 2.5 h. Vertical bars represent standard errors (N = 3 for isolated CT; N = 2 for herbage).

acetone, we reduced heating temperature to 70 °C, which is close to the boiling point of a 1:1 mixture of butanol and acetone.[19] Proper care was taken during the assay to prevent evaporative losses of acetone, which would bias absorption readings. As illustrated in Figure 1 for herbage CT and in Figure 2 for leaf CT, acetone addition increased color yields much more from CT-containing tissue than from isolated CT, and responses were greater for BT than for BFT. On the basis of R^2 values, quadratic response surface models of acetone content vs heating time explained 79−99% of the variation in color yields. Five of the eight CT sources had a nonsignificant lack of fit, indicating the quadratic response-surface model adequately described the data (see Tables 1S and 2S, Supporting Information). Lack of fit was significant for herbage and leaves from BT and for CT isolated from BFT leaves, indicating that more complex models or additional data points would be needed to more precisely define color responses, but the very high R^2 for most CT sources suggested the quadratic model adequately described the data for our purposes. Most CT sources had significant linear and quadratic regression parameters for heating time and acetone content, and the absence of an interaction for most CT sources indicated that both factors usually acted independently to influence color yields.

On the basis of response surface analyses, predicted maximum anthocyanidin color yields in the butanol−HCl−iron assay were obtained with acetone concentrations ranging from 30 to 58% and heating times ranging from 2.2 to 3.6 h (Figures 1 and 2). A plateau surrounding the predicted maximum value of each CT source suggested a variety of acetone concentrations, and heating time combinations could be used to produce near maximal color yields. Thus, calculations with parameter estimates (see Table 1S and 2S, Supporting Information) indicated a butanol−HCl−iron reagent containing 50% acetone with heating at 70 °C for 2.5 h would produce color yields at ∼99% of the predicted maximum value for six of the eight CT sources evaluated. For the remaining two sources (CT isolated from BFT herbage or leaves), these conditions produced slightly lower but acceptable color yields at ∼92% of the predicted maximum value. Because of slightly depressed color production, an acetone concentration of 50% might arguably be considered too high for CT isolated from BFT. We, however, opted to maximize anthocyanidin production from tissue and assumed that any reduction in color yield from a CT standard would reflect losses that also occurred during reactions with tissue. Therefore, we concluded that a butanol−HCl−iron reagent formulated with 50% acetone and 7% total water when heated at 70 °C for 2.5 h would provide suitable conditions for assaying CT in *Lotus*.

Next the requirement for iron(III) in the assay was determined by heating herbage and isolated herbage CT at 70 °C for 2.5 h in butanol−HCl reagents formulated with 0 to 0.6 mg mL^{-1} of ammonium iron(III) sulfate dodecahydrate, 0 to 60% acetone, and 7% total water. In the absence of acetone, anthocyanidin color yields from isolated CT increased 3-fold when iron was added to the reagent (Figure 3). Color yields from isolated CT were, however, low or erratic (as evidenced by large standard errors) if acetone was added to reagent lacking iron, but iron added with acetone increased and stabilized color yields. An analysis of variance indicated all iron salt additions of 0.2−0.6 mg mL^{-1} produced similar color yields from isolated CT. On the basis of visual observations, adding only iron or only acetone to the butanol−HCl reagent accelerated color formation from isolated CT, but for unknown reasons, adding acetone alone often led to a rapid fading of color after 15−30 min of heating. In the presence of iron, color production with acetone was slower, but peak color remained quite stable with extended heating (e.g., see Figure 2).

As observed in previous experiments, acetone increased anthocyanidin color yields from herbage, but the response and its reproducibility were not markedly influenced by the presence or absence of iron in the butanol–HCl assay (Figure 3). While needing confirmation, the lack of a response to added iron may be due to the presence of iron or other transition metal ions in plant tissue. Overall, our results indicate that butanol–HCl assays run with acetone must include added iron for extensive and stable anthocyanidin production from isolated CT, but added iron is not a requirement for assaying CT in tissues. For convenience and consistency, however, acetone–butanol–HCl reagent containing iron could be used in assays for both CT standards and tissue.

Finally, although initial work suggested cyclohexanone might be a promising cosolvent, subsequent studies revealed that butanol–HCl–iron reagents formulated with up to 60% cyclohexanone (or another cyclic ketone cyclopentanone) were less effective than acetone for maximizing anthocyanidin color yields from BT leaves (data not shown). At higher concentrations, cyclohexanone or especially cyclopentanone also depressed and badly distorted anthocyanidin peaks for CT isolated from BT, indicating that cyclic ketones were less useful than acetone as cosolvents for the butanol–HCl assay.

Quantifying Total CT with the Conventional vs Modified Assay. For this comparison, isolated CT standards and tissues were heated at 95 °C in a conventional assay utilizing butanol–HCl–iron reagent, but heating time was increased to 2.5 h to ensure maximal anthocyanidin color yields from tissue (see Figure 1S, Supporting Information). The modified assay utilized a similar butanol–HCl–iron reagent prepared with 50% acetone, with heating at 70 °C for 2.5 h; optimal conditions for the modified assay were ascertained from experiments described above.

As recommended,[1,2] CT standards were prepared from the plant materials under study by fractionating acetone/water extracts from BFT and BT leaves by Toyopearl HW-55F size-exclusion chromatography. Stepwise elution with 3:7 to 7:3 acetone/water yielded CT fractions that varied in purity from 40 to 99 g CT/100 g fraction as determined by thiolytic degradation with benzyl mercaptan (Table 2). The 7:3 acetone/water BFT fraction and the 1:1 acetone/water BT fraction selected as CT standards had respective purities of 99 and 93 g CT/100 g fraction, mean degrees of polymerization of 30 and 36, and PC:PD ratios of 60:40 and 21:79. On the basis of the data in Table 2, subjective selection of fractions or pooling of acetone/water fractions without chemical characterization could result in the use of CT standards of lower purity in butanol–HCl assays. Preliminary NMR studies suggest that "purified" CT fractions prepared by gel filtration with Toyopearl HW-55F or Sephadex LH-20 can be substantially contaminated with carbohydrates and phospholipids. Such contamination can occur even when crude extracts are extracted with organic solvents to remove nonpolar contaminants and columns are washed with aqueous methanol to try and elute carbohydrates and low molecular weight flavonoids prior to CT elution with acetone/water (W.E.Z. and I.M.-H., 2012, unpublished results). If not taken into account, such contamination would obviously depress anthocyanidin yields from standards and thereby inflate CT estimates in tissue. A sampling of recent studies[20−25] indicates researchers rarely if ever determine the actual purity of CT used as standards in butanol–HCl assays. Therefore, our results indicate the purity of CT standards must be characterized and taken into account when quantifying CT in plant samples by the butanol–HCl assay.

Table 2. Purity (g CT/100 g fraction), Ratio of Procyanidin (PC) to Prodelphinidin (PD) Units, and Mean Degree of Polymerization (mDP) of BFT and BT CT Fractions Eluted from Toyopearl HW-55F with Aqueous Acetone As Determined by Thiolysis

CT source	fraction (acetone/water)	% purity	PC to PD ratio	mDP
BFT	3:7	40.4	73:27	5.1
	1:1	62.2	65:35	10.8
	7:3	98.9	60:40	30.0
BT	3:7	53.8	22:78	38.0
	1:1	92.6	21:79	36.1
	7:3	68.7	16:84	ND[a]

[a]ND, not determined due to difficulty in detecting terminal units.

The isolated CT standards we selected gave slightly curvilinear anthocyanidin color yields when heated in the conventional assay (Figure 4). By comparison, heating in the modified assay with acetone gave slightly lower color yields for the BFT standard and higher yields for the BT standard. Overall, the results suggest that anthocyanidin production in the modified assay with acetone would be proportional to the quantity of CT present. By contrast, a curvilinear or biphasic response of CT standards has often been noted with the conventional assay,[13] but the reason for this problem is unknown.

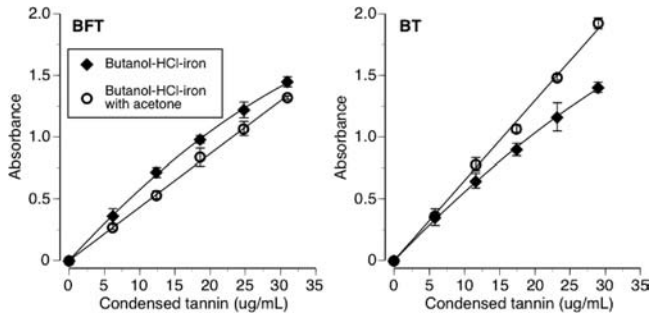

Figure 4. Absorbance by anthocyanidins produced from CT standards isolated from leaves of BFT and BT heated in the conventional butanol–HCl–iron reagent or the modified butanol–HCl–iron reagent amended with acetone. Vertical bars represent standard errors (N = 3).

Compared to the standard assay, the modified assay run with acetone–butanol–HCl–iron reagent increased total CT estimates by about 1.9-fold for BFT tissues and 2.5 to 3.2-fold for BT tissues (Table 3). Gains in measurable CT with the modified assay with acetone are particularly noteworthy for BT, because it contains a higher proportion of prodelphinidin-rich CT that are reported to be more resistant to depolymerization in butanol–HCl reagent and more difficult to solubilize from tissue than procyanidin-rich CT.[2] The wavelength of maximal anthocyanidin absorption was similar for CT standards and tissues, averaging 554 nm for BFT and 561 nm for BT. The difference in λ_{max} can be attributed to the differences in PC:PD ratios of the two samples where the liberated cyanidin and delphinidin exhibit $\Delta\lambda_{max}$ of 10 nm.[26] The use of the modified reagent with acetone resulted in a slight bathochromic shift of the λ_{max} by about 2–4 nm compared to the conventional

Table 3. Dry Matter Concentrations (mg g^{-1}) of CT in BFT and BT As Determined by a Standard Butanol−HCl−Iron Assay vs a Modified Acetone−Butanol−HCl−Iron Assay Run with Isolated CT Standards of Defined Purity (N = 3)

	BFT		BT	
assay	herbage	leaves	herbage	leaves
butanol−HCl	15.0 b[a]	30.8 b	12.5 b	47.0 b
acetone−butanol−HCl	29.3 a	57.1 a	40.0 a	117.9 a
standard error of the mean	2.50	2.78	1.32	4.97

[a]In each column, least square means followed by unlike letters differ at P = 0.05.

reagent. When quantifying CT, the use of CT-free tissue heated in butanol−HCl reagent is one of several approaches used for removing the absorbance of nonanthocyanidin degradation products from anthocyanidin peaks.[13] Our use of heated alfalfa solutions that lacked an anthocyanidin absorption peak reduced CT estimates for BFT and BT tissue by an average of only 1.5 mg g^{-1} of dry matter in both assays, suggesting that nonanthocyanidin degradation products caused minimal interference in the assays.

Gel NMR Analysis of CT in Tissue before and after Assays. In likely its first application to CT analysis, we used gel-state NMR spectroscopy[18] to demonstrate that butanol−HCl−iron reagent formulated with 50% acetone increased anthocyanidin color yields by facilitating complete solubilization of CT from *Lotus* tissue. We used BT leaves for these studies because it gave the greatest response to acetone in the butanol−HCl assays. Spectra were taken in 4:1 DMSO-d_6/pyridine-d_5, which was originally utilized for gel-NMR of plant cell walls.[18] This solvent had no adverse effects on CT; spectra collected in the DMSO-d_6/pyridine-d_5 solvent showed no significant changes in resonances after 1 week at room temperature and after 7 months of storage frozen at −20 °C (data not shown).

The ^{1}H−^{13}C HSQC NMR spectrum of the CT standard from BT is shown in Figure 5, along with the following cross-peak assignments (^{1}H and ^{13}C chemical shifts in ppm): H/C-4 (4.78, 35.7), H/C-3 (3.98, 71.1), H/C-2 (5.27, 75.0), H/C-6/8 (5.93, 95.8), H/C-2′/6′ (PD) (6.58, 105.6), H/C-2′/5′ (PC) (6.76, 115.0), and H/C-6′ (PC) (6.77, 117.3). Peak assignments were made according to previously reported ^{1}H, ^{13}C, and ^{1}H−^{13}C HSQC assignments for CT in acetone-d_6, acetone-d_6/D_2O mixtures, or DMSO-d_6.[27−33] Overall, NMR resonances were broad due to the polymeric nature and the 2,3- and 3,4-cis and -trans orientations and atropisomerism of CT. Thus, we only made assignments for the predominant PC and PD structures and have not, for the time being, distinguished between cis and trans orientations in the flavanol C-ring.

Comparison of ^{1}H−^{13}C correlation HSQC NMR spectra of BT leaves ground to pass through a 0.5 mm screen of a cyclone mill (not shown) and after ball-milling (Figure 6) showed no significant differences, indicating that ball-milling did not modify or enhance CT analysis by gel-state NMR to any appreciable extent. Thus, high-quality ^{1}H−^{13}C HSQC NMR spectra can be collected on DMSO-d_6/pyridine-d_5 gels prepared from either finely ball-milled or more coarsely ground plant material. Definitive CT structures that were clearly resolved and not obscured by other BT leaf components included cross-peaks for H/C-4, H/C-2, and H/C-6/8 in PC/PD units and the H/C-2′/6′ in PD units. Cross-peaks for H/C-2′/5′ and H/C-6′ from PC units were also tentatively identified but were likely comingled with resonances arising from other plant components such as guaiacyl and coumaroyl units in lignin.[18]

The ^{1}H−^{13}C HSQC NMR spectrum of BT leaf residue recovered after the conventional assay with butanol−HCl−iron reagent still contained prominent CT cross-peak resonances corresponding to the H/C-6/8 cross-peak of the A-ring and the H/C-2′/6′ of the B-ring (Figure 7a), whereas H/C-4 and H/C-2 cross-peak resonances appeared weak or were not observed (spectral area not shown). By contrast, no CT resonances were detected in the spectrum of residue recovered after reaction with acetone−butanol−HCl−iron reagent (Figure 7b). On the basis of a serial dilution study, the detection limit of H/C-6/8 and H/C-2′/6′ resonances was 0.125 mg of CT (data not shown). This is less than 1% of the estimated 15.5 mg of CT originally contained in the 132 mg of BT leaves needed to produce the 50 mg of assay residues analyzed by HSQC NMR. Thus, unlike the conventional reagent, the modified butanol−HCl−iron reagent with acetone achieved essentially complete degradation of CT in BT leaf tissue.

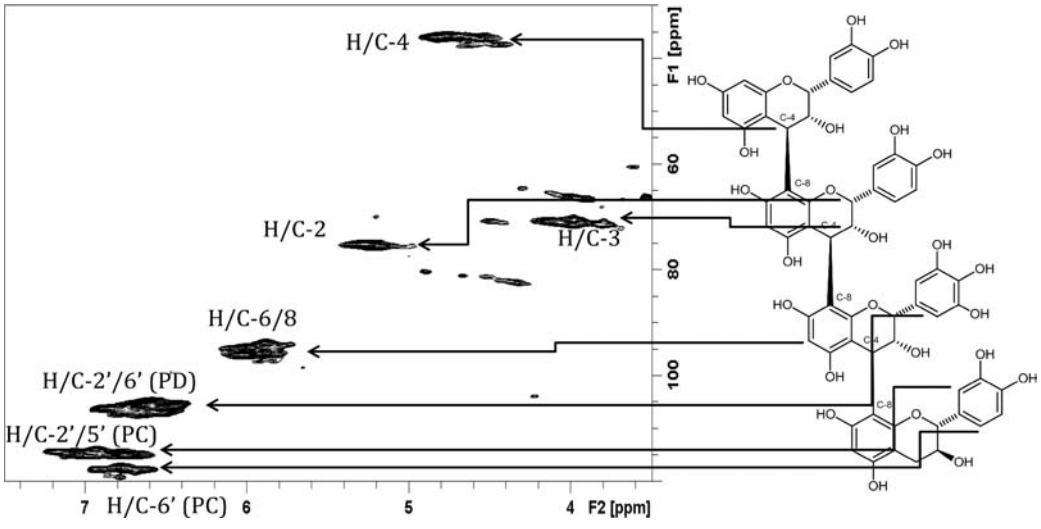

Figure 5. The ^{1}H−^{13}C HSQC NMR spectrum of a CT standard isolated from BT leaves. See the text for a discussion of the assignments.

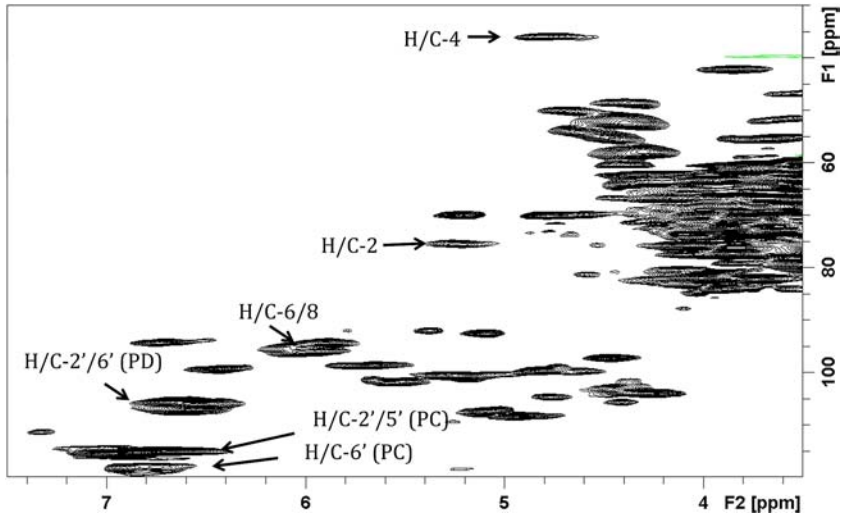

Figure 6. $^{1}H-^{13}C$ HSQC NMR spectrum of BT leaves after ball milling. Resonances due to CT are indicated.

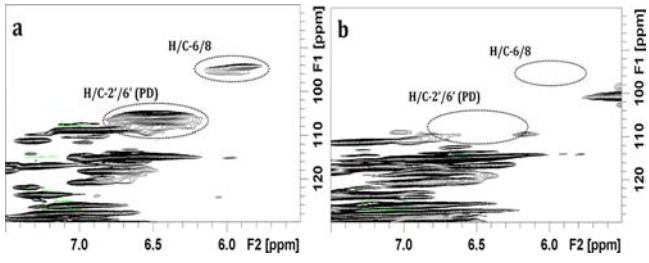

Figure 7. $^{1}H-^{13}C$ HSQC NMR spectra of ball-milled residues of BT leaves recovered following heating in conventional butanol–HCl–iron reagent (a) and modified butanol–HCl–iron reagent with 50% acetone (b). Both spectra were obtained with identical quantities of residue and under identical spectrometer acquisition parameters.

The impact of adding acetone to the butanol–HCl–iron reagent is also evident in stacked ^{13}C NMR gel spectra (Figure 8) of the CT standard from BT leaves (A), ball-milled BT leaf tissue (B), and ball-milled residues collected after the conventional assay (C) and the modified assay with acetone (D). On the basis of published ^{13}C NMR assignments,[27,30] resonances arising from C3′/C5′ carbons of PD units and the C3′/C4′ carbons of PC units at 144–146 ppm as well as other CT structural features were clearly evident for all spectra except for residues collected after the modified assay with acetone. Using solid-state ^{13}C NMR spectroscopy, Makkar et al.[14] also found that the conventional butanol–HCl–iron reagent formulated with 10% acetone (included not to evaluate its cosolvent effects, but because assays are often run with aqueous acetone extracts) incompletely removed CT from plant tissue, even after repeated treatment. We, however, show for the first time that a single treatment with butanol–HCl–iron reagent formulated with 50% acetone completely removed CT from plant tissue. Not coincidentally, it has also been noted that acidified acetone is especially effective for extracting CT that are strongly bound to fiber.[2]

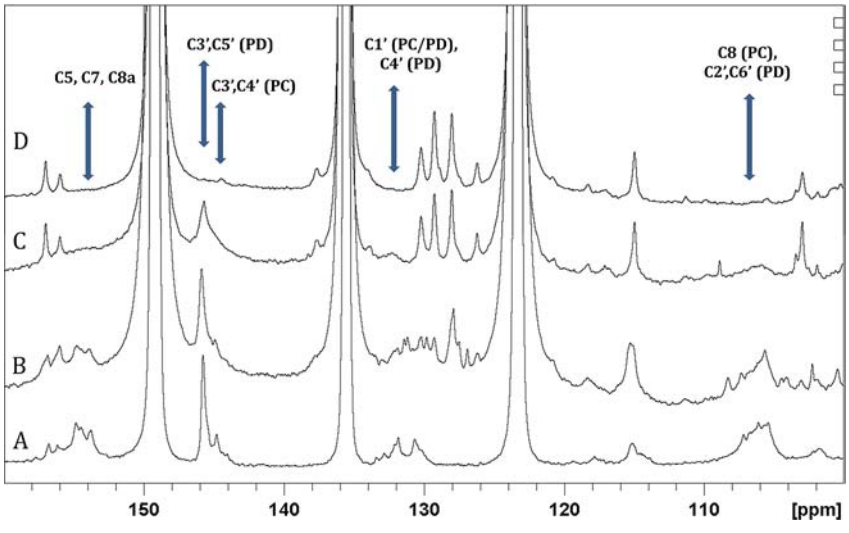

Figure 8. Stacked ^{13}C spectra showing CT resonances from BT for an isolated CT standard (A), ball-milled leaves (B), and ball-milled leaf residues recovered following heating in conventional butanol–HCl–iron reagent (C) or in modified butanol–HCl–iron reagent with 50% acetone (D). CT resonances are indicated above spectrum D. The three large peaks in the spectrum are due to ^{13}C resonances from pyridine in the 4:1 DMSO-d_6/pyridine-d_5 NMR solvent. Spectra C and D were obtained with identical quantities of residue and under identical spectrometer acquisition parameters.

In conclusion, an initial screening of various cosolvents revealed that acetone was particularly effective for increasing anthocyanidin color yields from BFT and BT tissue by the butanol−HCl−iron assay. Subsequent studies revealed that color responses to added acetone were greater for tissue than for isolated CT and greater for PD-rich CT in BT than for PC-rich CT in BFT. Nevertheless, butanol−HCl−iron reagent formulated with 50% acetone and 7% total water with heating at 70 °C for 2.5 h produced near maximal color yields from all CT sources. Reagent prepared with acetone required added iron(III) for stable anthocyanidin production from isolated CT, but not from tissue. A modified acetone−butanol−HCl−iron assay gave linear responses with *Lotus* CT standards and increased determinations of total CT in herbage and leaves by 1.85−3.2-fold over the conventional assay run without acetone. Quantitation was further improved by the use of thiolysis to identify and define the purity of CT fractions used as standards. Gel-state ^{13}C and $^{1}H-^{13}C$ HSQC NMR spectra of isolated CT, intact tissue, and insoluble residues collected after butanol−HCl assays revealed that acetone addition increased anthocyanidin yields by facilitating complete solubilization of CT from tissue. Now that the benefits of added acetone have been established, our ongoing work is aimed at further refining the acetone−butanol−HCl assay and assessing its analytical performance on a wider array of plant materials, both by direct analysis of total CT and by sequential analysis of extractable and bound CT. Studies aimed at more fully understanding the mechanism by which acetone improves anthocyanidin yields by the butanol−HCl assay are also currently underway.

■ ASSOCIATED CONTENT

Supporting Information

Figure 1S (anthocyanidin color yields from isolated CT and herbage of BFT and BT as influenced by heating time at 95 °C in conventional butanol−HCl−iron reagent) and Tables 1S and 2S (response surface parameters for anthocyanidin production from isolated CT and tissues of BFT and BT herbage and leaves when heated at 70 °C for 0.5 to 3 h in butanol−HCl−iron reagent amended with 0−60% of acetone). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: john.grabber@ars.usda.gov. Tel: 608 890 0059. Fax: 608 890 0076.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

BFT, birdsfoot trefoil; BT, big trefoil; CT, condensed tannin(s); mDP, mean degree of polymerization; PC, procyanidin; PD, prodelphinidin.

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