Selection of a Method of Condensed Tannin Analysis for Studies with Rumen Bacteria†

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Four colorimetric methods, vanillin-HCl, Prussian blue, 1-butanol-HCl, and H_2SO_4, were tested for their suitability for measurement of condensed tannins in media used for the culture of rumen bacteria. Of the four, only the H_2SO_4 procedure was suitable for quantifying condensed tannins in culture media at concentrations used in studies with rumen bacteria. Water reduced the extent of chromophore development in both the vanillin-HCl and 1-butanol-HCl methods. Components in the culture medium resulted in substantial color development in the absence of condensed tannins in both the vanillin-HCl and Prussian blue methods. In contrast, the H_2SO_4 method was not subject to a loss in chromophore yield from water or to interference from medium components. The chromophore formed was stable, and the assay enabled microgram quantities of condensed tannins to be measured in microbial medium. Condensed tannins in condensed tannin-bovine serum albumin complexes were readily quantified by the H_2SO_4 method. Thus, the potential exists for the H_2SO_4 method to quantify condensed tannins in both condensed tannin-bacterial cell and condensed tannin-bacterial enzyme complexes.

INTRODUCTION

Condensed tannins reduce the voluntary intake and the digestibility of forages by ruminants (Palo, 1985; Windham et al., 1990; Waghorn et al., 1990). Researchers have attributed this tannin-induced reduction in voluntary intake to a decrease in the palatability of the forage (Kumar and Singh, 1984) and the accumulation of undigested feed in the rumen as a result of the inhibitory effects of condensed tannins on microbial fermentation (Waghorn et al., 1990). Palo (1985) speculated that condensed tannins may be toxic to rumen microorganisms or inhibit their access to nutrients. Condensed tannins have been shown to alter the products of microbial fermentation (Sadanandan and Arora, 1979) and to reduce the activity of microbial enzymes (Lyford et al., 1967; Makkar et al., 1988, 1990). However, experiments to examine the direct effects of condensed tannins on rumen microorganisms have not been reported.

A major impediment to studying the effects of condensed tannins on rumen microorganisms has been the inability to rapidly and accurately quantify condensed tannins in microbial cultures. Present methods for the analysis of condensed tannins are essentially of two types, protein-binding assays and chemical (colorimetric) assays. Protein-binding assays have been used to quantify condensed tannins in forages (Hagerman and Butler, 1978; Makkar, 1989) and to estimate their effects on biological activity (Robbins et al., 1987). The extent of insoluble complex formation between condensed tannins and proteins is a characteristic of both the condensed tannins and the proteins. Furthermore, the affinity of condensed tannins for proteins and carbohydrates varies with pH (Oh and Hoff, 1987). The protein component and the pH of the culture fluid change continuously during fermentation, which renders protein-binding assays unsuitable for the measurement of condensed tannins in microbial cultures.

Numerous colorimetric assays for condensed tannins have been developed, including Folin-Denis (Folin and Denis, 1912; Swain and Hills, 1959), Prussian blue (Price and Butler, 1977), vanillin-HCl (Broadhurst and Jones, 1978), 1-butanol-HCl (Porter et al., 1986), and H_2SO_4 (Bate-Smith and Rasper, 1969). Folin-Denis reagent reacts with proteins and amino acids and was modified by Lowry et al. (1951) for routine protein analyses. As culture fluid contains both proteins and amino acids, the Folin-Denis method is unsuitable for the measurement of condensed tannins in this application. The objective of the present study was to evaluate four colorimetric assays for their ability to quantify condensed tannins in water and culture medium.

MATERIALS AND METHODS

Extraction of Condensed Tannins. Condensed tannins were obtained from alfalfa (*Medicago sativa* L.) seed, sainfoin (*Onobrychis viciifolia* Scop.) leaves, and fresh, whole birdsfoot trefoil (*Lotus corniculatus* L.) foliage, as described by Koupai-Abyazani et al. (1992). Plant material was homogenized with 70% acetone containing 0.1% (w/v) ascorbic acid in a Waring blender. Acetone extracts were reduced to the aqueous phase by evaporation under reduced pressure at 35 °C. The resulting aqueous phase was extracted with petroleum ether followed by ethyl acetate until both organic solvents were clear. Traces of ethyl acetate were removed from the remaining aqueous fraction by rotary evaporation. The resulting fraction was diluted 1:1 with methanol and applied to a Sephadex LH-20 column equilibrated in 50% aqueous methanol. Columns were then washed with 50% aqueous methanol until the eluate was clear. Condensed tannins were eluted from the columns with 70% acetone and evaporated to the aqueous fraction. The aqueous phase was lyophilized, and the extracted condensed tannins were stored in the dark, in a desiccator at 5 °C. Extracts were examined by proton and carbon NMR spectroscopy and were found to produce spectra indicative of high molecular weight polyphenolic compounds. Condensed tannins from birdsfoot trefoil were used to compare the four colorimetric methods. Further investigations of the H_2SO_4 method used condensed tannins from all three plant sources.

Preparation of Standards. Three stock solutions (1.0 mg/mL) of condensed tannins from each source (birdsfoot trefoil,

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Table I. Composition of Modified Scott and Dehority's Medium[a]

component	amount, mg/100 mL	component	amount, mg/100 mL
KH_2PO_4	90.0	resazurin	0.1
NaCl	90.0	L-cysteine·HCl·H_2O	100.0
$CaCl_2$	5.0	pyridoxine hydrochloride	0.2
$MgSO_4$	5.0	riboflavin	0.2
$MnSO_4·H_2O$	2.0	thiamin hydrochloride	0.2
$FeSO_4·7H_2O$	2.0	nicotinamide	0.2
$ZnSO_4·7H_2O$	2.0	calcium D-pantothenate	0.2
Na_2CO_3	400.0	*p*-aminobenzoic acid	0.01
acetic acid	133.0	folic acid	0.005
isobutyric acid	6.6	biotin	0.005
isovaleric acid	8.0	B_{12}	0.0005
valeric acid	8.0		

[a] Also contained 10% (v/v) autoclaved, clarified rumen fluid.

sainfoin leaves, and alfalfa seed) were prepared by dissolving them in methanol, water, and the liquid medium of Scott and Dehority (1965; Table I), modified to contain autoclaved, clarified rumen fluid (10% v/v). The composition of this medium is presented in Table I. Stock solutions were then diluted with like diluent to give final concentrations of 20–100 μg of condensed tannin in the final reaction mixture. Reaction mixtures were prepared in triplicate for each concentration of condensed tannin. Catechin was obtained from Sigma Chemical Co. (St. Louis, MO) and tested for chromophore formation in the H_2SO_4 method.

Colorimetric Assays. Four colorimetric assays were tested for their ability to quantify birdsfoot trefoil condensed tannins in each of the above solvents. For each assay, negative controls were prepared by substituting solvent which contained no condensed tannins for the standard sample. All absorbencies were measured on a Gilford Model III spectrophotometer (Oberlin, OH).

Vanillin–HCl. Samples were analyzed as described by Price et al. (1978) except that 4% (w/v) vanillin (Sigma Chemical Co.) in methanol was used, as described by Broadhurst and Jones (1978). Reagent was prepared by mixing equal volumes of 4% (w/v) vanillin in methanol and 8% (v/v) concentrated HCl in methanol. Reaction mixtures consisted of 0.1 mL of standard, 2 mL of methanol, and 5 mL of the vanillin–HCl reagent. Following mixing, tubes were incubated for 15 min in a water bath at 30 °C and the absorbance was measured at 495 nm.

The effect of water on this assay was studied by substituting 0.5, 2.5, 5.0, 7.5, and 10.0% (v/v) water for methanol in the reaction mixture. Samples were prepared by adding 0.1 mL of standard so that the final 2.0 mL of reaction mixture contained 150 μg of condensed tannins.

Prussian Blue. A modification of the method of Price and Butler (1977) was assessed using birdsfoot trefoil condensed tannins. Ferric chloride (0.1 M) and $K_3Fe(CN)_6$ (8 mM) were prepared in 0.1 M HCl. Reaction mixtures contained 0.1 mL of standard, 0.5 mL of $FeCl_3$, and 0.5 mL of $K_3Fe(CN)_6$. The solution was mixed, diluted with 8 mL of distilled water, and allowed to stand at room temperature for 10 min. Absorbance of the samples was measured at 720 nm.

1-Butanol–HCl. The procedure of Porter et al. (1986) was used to analyze birdsfoot trefoil condensed tannins dissolved in methanol, water, and medium. Reaction mixtures contained 0.2 mL of standard, 2.4 mL of 1-BuOH/concentrated HCl (95:5 v/v), and 80 μL of ferric reagent [2% (w/v) $NH_4Fe(SO_4)_2·12H_2O$ in 2 M HCl] in thick-walled 16 × 100 mm culture tubes. Tubes were sealed with Teflon-lined caps and heated in a water bath at 95 °C for 40 min. Samples were removed from the bath and cooled to room temperature, and absorbance of the solution was measured at 557 nm.

H_2SO_4. Bate-Smith and Rasper (1969) originally described a method which used 43% (v/v) H_2SO_4 in methanol for the analysis of condensed tannins. Reaction mixtures contained 2.0 mL of concentrated H_2SO_4 with 0.1 mL of standard. Absorption curves (400–800 nm) of the color complex were recorded at room temperature using 60 μg of birdsfoot trefoil condensed tannins dissolved in water, methanol, medium, and 100% clarified rumen fluid. Completion of the reaction and stability of the color complex were evaluated by measuring the absorbance of reaction mixtures after 1, 2, 8, and 24 h. For subsequent assays, mixtures were allowed to react at room temperature for 1 h and absorbance was measured at 580 nm. Calibration curves were generated for condensed tannins from birdsfoot trefoil, sainfoin leaves, and alfalfa seed coat.

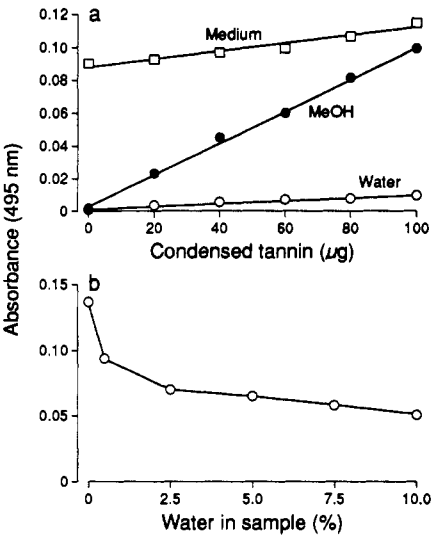

Figure 1. (a) A_{495} for the vanillin–HCl method for condensed tannins from birdsfoot trefoil dissolved in either water, methanol, or rumen medium using the vanillin–HCl method. (b) Effect of water on the sensitivity of the vanillin–HCl method.

Bovine serum albumin (BSA; Sigma) was used in evaluating the ability of the H_2SO_4 method to measure condensed tannins complexed with protein. Condensed tannin–BSA complexes were formed by combining 1.0 mL of BSA solution (0.5–2.0 mg/mL BSA in 0.2 M sodium acetate buffer, pH 5.0) with 0.1 mL of birdsfoot trefoil condensed tannins (300 μg/mL). After 15 min, complexes were collected by centrifugation (15600*g*, 5 min, 4 °C). Both the pellet and the supernatant were retained. Condensed tannins remaining in the supernatant were determined as described above. Condensed tannin–BSA complexes were washed twice with 1.0 mL of 0.2 M sodium acetate buffer (pH 5.0) and centrifuged (15600*g*, 5 min, 4 °C). Complexes were suspended in 1.0 mL of 0.2 M sodium acetate buffer (pH 5.0), maintained at 4 °C, and sonicated (30 s, 45% output, Sonic 300 Dismembrator, Artek Systems Corp., Farmingdale, NY) to form a homogenate. While stirring, a 0.1-mL aliquot was removed, and the amount of condensed tannins associated with the precipitate was determined using the H_2SO_4 method.

RESULTS AND DISCUSSION

Vanillin–HCl. Vanillin–HCl analysis of aliquots of medium containing birdsfoot trefoil condensed tannins produced solutions with high background absorbencies ($Y = 0.002X + 0.088$, $R^2 = 0.998$; Figure 1a). The development of color in the absence of condensed tannins suggests that non-tannin, vanillin-positive compounds are present in the medium. Subtraction of the absorbance of the zero tannin samples resulted in a calibration curve similar to that observed when vanillin–HCl was reacted with condensed tannins dissolved in water [$Y = (8.2 \times 10^{-5})X + 0.002$, $R^2 = 0.998$]. Dissolving condensed tannins in water lowered the yield of chromophore as compared to condensed tannins dissolved in methanol ($Y = 0.0009X + 0.004$, $R^2 = 0.945$; Figure 1a). This observation led us to investigate the effects of water on the vanillin–HCl assay. Concentrations of water as low as 0.5% in methanol caused a 31% decrease in absorbance as compared to condensed tannins dissolved in 100% methanol (Figure 1b). The reaction of the vanillin–HCl reagent with non-tannin components of the medium, combined with a reduction in chromophore yield in the presence of water, makes the

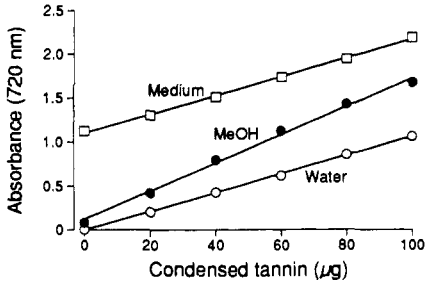

Figure 2. A_{720} for condensed tannins from birdsfoot trefoil dissolved in either water, methanol, or rumen medium using the Prussian blue method.

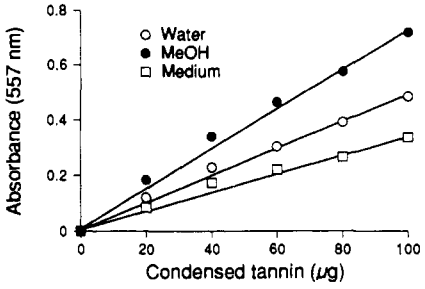

Figure 3. A_{557} for condensed tannins from birdsfoot trefoil dissolved in either water, methanol, or rumen medium using the 1-butanol–HCl method.

vanillin–HCl method unsuitable for studies with rumen microorganisms.

Prussian Blue. The yield of chromophore in the Prussian blue method, unlike that in the vanillin–HCl method, was not seriously reduced by the presence of water ($Y = 0.011X - 0.004$, $R^2 = 0.999$) and readily measured condensed tannins dissolved in methanol ($Y = 0.016X + 0.115$, $R^2 = 0.995$, Figure 2). However, as indicated by the absorbance of the blank, the Prussian blue method was subject to interference from other non-tannin compounds present in the medium ($Y = 0.011X + 1.103$, $R^2 = 0.998$, $n = 16$; Figure 2). The Prussian blue method measures total phenolics and is based on the reduction of the ferric to the ferrous ion and the formation of a ferricyanide–ferrous ion complex (Price and Butler, 1977). Thus, the Prussian blue method is not specific to condensed tannins, and the presence of compounds that are easily oxidized can result in a false positive test (Hagerman and Butler, 1989). The concentration of non-tannin phenolics in the medium is likely to change during fermentation, and readily oxidizable compounds such as cysteine hydrochloride are necessary for maintenance of anaerobiosis. Price and Butler (1977) recommended a salt extraction to determine the amount of non-tannin phenolics that contribute to color development. However, this procedure is time-consuming and is unlikely to extract all of the non-tannin compounds that contribute to the development of the Prussian blue color complex in anaerobic medium.

1-Butanol–HCl. In contrast to the vanillin–HCl and Prussian blue methods, background absorbance in the 1-butanol–HCl method was no higher in medium ($Y = 0.003X + 0.018$, $R^2 = 0.984$, $n = 16$) than in water ($Y = 0.005X + 0.018$, $R^2 = 0.994$, $n = 16$) or methanol ($Y = 0.007X + 0.031$, $R^2 = 0.992$, $n = 16$; Figure 3). However, the chromophore yield of this method was reduced both by water and by medium as compared to methanol. Heating condensed tannins in 1-butanol–HCl causes the autoxidation and cleavage of interflavenoid bonds and the release of anthocyanidins (Porter et al., 1986). Apparently, the addition of water and medium to the 1-butanol–HCl assay increases the occurrence of competing

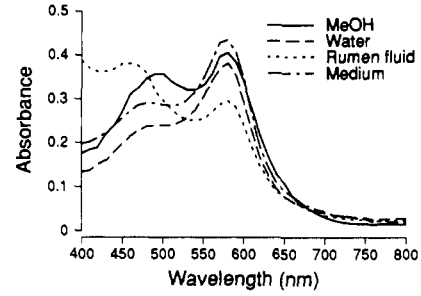

Figure 4. Absorption curves for birdsfoot trefoil condensed tannins dissolved in either water, methanol, 100% clarified rumen fluid, or rumen medium.

side reactions and decreases the yield of anthocyanidins. Although Porter et al. (1986) suggested that lyophilization would correct interference due to water, this is not a practical solution for microbiological studies which generate large numbers of aqueous samples. Furthermore, other medium components, not removable by lyophilization, may also contribute to a reduction in the chromophore yield of the 1-butanol–HCl assay. Condensed tannins readily form complexes with both proteins and carbohydrates (Davis and Hoseney, 1979; Leinmuller et al., 1991), and it is likely that these reactions occur when condensed tannins are mixed with the medium. If the 1-butanol–HCl method does not disrupt these complexes, the condensed tannins they contain would escape detection. This would account for the further reduction in chromophore yield observed when 1-butanol–HCl was used to measure condensed tannins in medium over those dissolved in water or methanol. A method that quantifies the condensed tannins in condensed tannin–protein or condensed tannin–carbohydrate complexes would be preferable for microbiological studies.

H_2SO_4. Condensed tannins dissolved in water, methanol, or medium, formed a bluish purple chromophore with λ_{max} of 580 nm when reacted with concentrated H_2SO_4 (Figure 4). When this assay was used to analyze condensed tannins dissolved in clarified rumen fluid, absorption between 400 and 500 nm was increased, but a distinct absorbance peak at 580 nm was still evident. Bate-Smith and Rasper (1969) found that 50% H_2SO_4 substantially increased the absorption at 465 nm and thus elected to use 43% H_2SO_4 in their procedure. We found that although concentrated H_2SO_4 increased absorption between 450 and 500 nm, it also enhanced the yield of the chromophore at 580 nm. Since absorption at 450 and 500 nm did not appréciably interfere with absorption of the chromophore at 580 nm, we chose to use concentrated H_2SO_4.

As with the 1-butanol–HCl method, production of a bluish purple chromophore in the presence of H_2SO_4 is consistent with the oxidative cleavage of condensed tannins and the release of anthocyanidins. In fact, the apparent similarity in the 1-butanol–HCl and H_2SO_4 methods, combined with the overwhelming popularity of the 1-butanol–HCl assay, has led to an under-recognition of the H_2SO_4 method, as evidenced by the omission of this method in several review papers (Temple, 1982; Mangan, 1988; Hagerman and Butler, 1989; Leinmuller et al., 1991). However, the differences in λ_{max} between the H_2SO_4 (580 nm) and 1-butanol–HCl (557 nm) methods, and the fact that the chromophore yield for H_2SO_4 method was similar for condensed tannins dissolved in water ($Y = 0.008X - 0.003$, $R^2 = 0.999$), methanol ($Y = 0.007X - 0.003$, $R^2 = 0.999$), and medium ($Y = 0.008X - 0.003$, $R^2 = 0.996$; Figure 5a), indicate that the reactions in these two assays are not

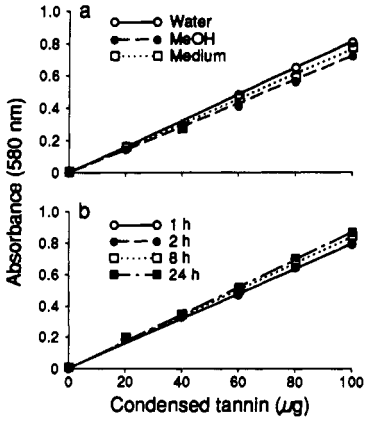

Figure 5. (a) A_{580} for condensed tannins from birdsfoot trefoil dissolved in either water, methanol, or rumen medium using the H_2SO_4 method. (b) A_{580} for the H_2SO_4-generated color complex after standing under fluorescent light at room temperature for 1, 2, 8, or 24 h.

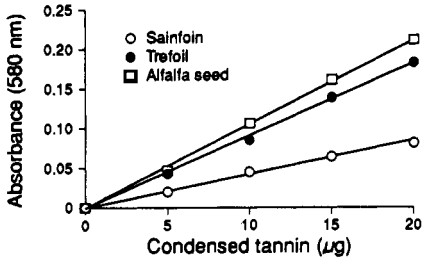

Figure 6. A_{580} for condensed tannins from sainfoin, alfalfa seed coat, and birdsfoot trefoil using the H_2SO_4 method.

identical. The observation that medium did not adversely affect chromophore yield and that color did not develop in the medium blank encouraged us to investigate further the suitability of the H_2SO_4 method for experiments in rumen microbiology.

The chromophore formed from condensed tannins in the H_2SO_4 assay was stable even after 24 h of exposure to fluorescent lighting at room temperature (Figure 5b). Color development was rapid and complete after 15 min at room temperature. This is in contrast to the vanillin–HCl chromophore, which is sensitive to light (Broadhurst and Jones, 1978), and the 1-butanol–HCl method, which requires incubation at 95 °C for 40 min to form a color complex (Porter et al., 1986).

Analysis of condensed tannins extracted from birdsfoot trefoil, sainfoin leaves, and alfalfa seed coat and catechin produced three distinct calibration curves ($Y = 0.001X - 0.002$, $R^2 = 0.998$, $Y = 0.004X + 0.001$, $R^2 = 0.996$, $Y = 0.001X - 0.003$, $R^2 = 0.998$, respectively) and no detectable color with catechin (Figure 6). Maxson and Rooney (1972) cited inadequate standards and differing absorption maxima as serious limitations of the original H_2SO_4 method. Differences in calibration curves and absorption maxima are a result of variation in the types of anthocyanidins released from condensed tannin polymers (Mole and Waterman, 1987). These presumed limitations are not unique to the H_2SO_4 assay, and both the vanillin–HCl (van Hoven and Furstenburg, 1992) and 1-butanol–HCl (Wisdom et al., 1987) methods produce distinct calibration curves specific to the source of condensed tannins. Additionally, catechin and tannic acid, compounds that are routinely used as standards in condensed tannin analyses, are inadequate since they lack many of the properties of condensed tannins (Hagerman and Butler, 1989; Mole and Waterman, 1987). More recently, the use of isolated condensed tannins as standards for condensed

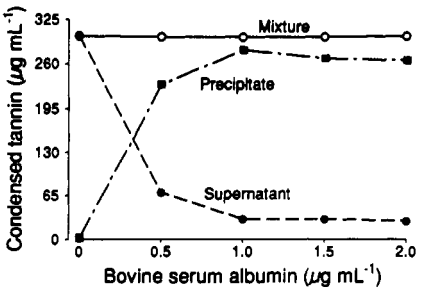

Figure 7. Measurements of condensed tannins in the supernatant and in condensed tannin–bovine serum albumin complexes by the H_2SO_4 method.

tannin analyses has been recommended (van Hoven and Furstenburg, 1992; Wisdom et al., 1987; Hagerman and Butler, 1989). This avoids the assumptions inherent to the use of catechin or tannic acid equivalents as indices of condensed tannin content. In the H_2SO_4 method, the lack of chromophore formation with catechin is a distinct advantage, since it permits the distinction between protein-precipitating polymers and non-protein-precipitating monomers.

The antimicrobial properties of condensed tannins have been attributed to their ability to form complexes with both the surface of the bacterial cell and bacterial enzymes (Lamed et al., 1987; Bae et al., 1993). Since this protein-complexing property of condensed tannins is of key interest to researchers in rumen microbiology, the H_2SO_4 method was studied further to determine if condensed tannins in tannin–protein complexes could be quantified. Addition of condensed tannins (300 μg/mL) to sodium acetate buffer containing increasing concentrations of BSA resulted in a progressive shift in the recovery of condensed tannins from the supernatant to the precipitate (Figure 7). Those condensed tannins capable of forming tannin–protein complexes appeared to be completely associated with BSA at 1 mg/mL, since further increases in the concentration of BSA failed to remove more condensed tannin from solution. At all concentrations of BSA, recovery of condensed tannins from the supernatant together with the precipitate was approximately 100% of the original condensed tannin concentration of 300 μg/mL. Condensed tannins bind with proteins through both hydrogen bond formation and hydrophobic interactions (Oh and Hoff, 1987). Complete recovery of condensed tannins from condensed tannin–BSA complexes indicates that H_2SO_4 disrupts these interactions and facilitates quantification of condensed tannins in these complexes.

CONCLUSION

Of the condensed tannin assays examined, only the H_2SO_4 procedure was suitable for measurement of condensed tannins in rumen medium. Water reduced the yield of chromophore in both the vanillin–HCl and 1-butanol–HCl assays. Both the vanillin–HCl and Prussian blue assays were subject to interference from components of media used for the culture of rumen bacteria. In contrast, the H_2SO_4 method was not subject to a reduction in the yield of chromophore with water as a solvent or to interference from medium components. The chromophore formed was not sensitive to light, and the assay was suitable for quantifying condensed tannins at concentrations commonly used for microbiological studies.

Condensed tannins in condensed tannin–protein complexes were readily quantified using the H_2SO_4 method. Recent work in our laboratory has shown that the H_2SO_4 assay is equally suited to quantifying condensed tannins

associated with rumen bacteria (Bae, unpublished data) and rumen fungi (McAllister, unpublished data). Thus, the potential exists to evaluate condensed tannins for their ability to form complexes with proteins on the surface of microbial cells and with extracellular enzymes. The ability of the H_2SO_4 method to dissociate condensed tannin–protein complexes and to quantify condensed tannins contained therein, combined with its rapidity, makes it an ideal method for the initial screening of rumen microorganisms for their ability to degrade condensed tannins. Studies are currently being conducted to evaluate the effectiveness of the H_2SO_4 method in the measurement of condensed tannins in the microbial, feed, and fluid fractions of rumen contents.

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