Due to excessive nonprotein N (NPN) formation by plant and ruminal proteases, only 10 to 30% of the crude protein (CP) originally present in alfalfa (*Medicago sativa* L.) undergoes intestinal digestion and absorption by ruminant livestock (National Research Council, 2001; Peltekova and Broderick, 1996). Although NPN conversion to microbial protein provides an important source of dietary protein for ruminants, excess NPN—accounting for up to one-third of alfalfa protein—is excreted in urine in forms that can be readily lost to the environment (Misselbrook et al., 2005; Yu et al., 2003). Excessive NPN formation is also a problem because degradation of high quality soluble proteins during wilting, ensiling, and ruminal fermentation leaves nutritionally inferior membrane proteins for gastrointestinal digestion (Hristov and Sandev, 1998; Kohn and Allen, 1995; Makoni et al., 1993, 1994). To alleviate poor protein utilization and animal performance, alfalfa is often supplemented with or substituted by other feeds possessing more desirable protein degradability characteristics. This approach, however, can exacerbate urinary excretion of N, increase feed costs, impair animal health due to inadequate

**Mechanical Maceration Divergently Shifts Protein Degradability in Condensed-Tannin vs. α-Quinone Containing Conserved Forages**

John H. Grabber*

Conditioning and conservation methods may interact with polyphenols to alter forage crude protein (CP) solubility and degradability. In this study, forages with ~200 g CP kg⁻¹ dry matter were roll conditioned or macerated, conserved as hay or silage, and then analyzed for CP fractions. Shifting from roll conditioning to maceration of polyphenol-free alfalfa (*Medicago sativa* L.) reduced buffer soluble protein (SP) with little effect on protease rumen-undegradable protein (RUP) and intestinal available protein (IAP, RUP minus acid-detergent insoluble CP). In birdsfoot trefoil (*Lotus corniculatus* L.), condensed tannin (CT) and maceration independently reduced SP and increased RUP to yield up to 62% more IAP in hay and 145% more IAP in silage than alfalfa. Based on results with trefoil, roll-conditioned forage with 70 to 120 g CT kg⁻¹ CP or macerated forage with more modest CT levels should meet a 350 g RUP kg⁻¹ CP target to support 35 kg d⁻¹ milk yield by cattle. In roll-conditioned red clover (*Trifolium pratense* L.), α-quinones formed by polyphenol oxidase reduced SP and increased RUP to yield 50% more IAP in hay and 88% more IAP in silage than alfalfa. Surprisingly, maceration reduced SP, RUP, and IAP in clover. Following maceration, RUP and IAP in conserved clover and trefoil responded similarly to CT, suggesting that maceration disabled α-quinone protection of protein substrates. Although red clover has high RUP, low reported milk yields indicate α-quinones might depress IAP.

U.S. Dairy Forage Research Center, USDA-ARS, Madison, WI 53706. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the USDA and does not imply its approval to the exclusion of other products that may also be suitable. Received 27 Aug. 2007. *Corresponding author (John.Grabber@ars.usda.gov).

**Abbreviations:** CP, crude protein; CT, condensed tannin; DM, dry matter; IAP, intestinal available protein; IRDP, insoluble rumen-degradable protein; NPN, nonprotein N; RDP, rumen-degradable protein; RUP, rumen-undegradable protein; SP, soluble protein.
fiber intake, or increase reliance on high-input row crops. Therefore, increasing the proportion of alfalfa protein digested in the gastrointestinal tract should improve the performance and sustainability of farming systems based on ruminant livestock.

In the future, the expression of protein–binding polyphenols such as condensed tannins (CT) and o-quinones in alfalfa should provide a sustainable approach for reducing wasteful proteolysis and increasing intestinal protein digestion by livestock (Sullivan and Hatfield, 2006; Xie et al., 2006). Direct ensiling studies have demonstrated that polyphenols in forage legumes can reduce proteolysis by up to 60% compared to alfalfa (Albrecht and Muck, 1991; Jones et al., 1995b). Studies with freeze-dried herbage also indicate that forage legumes with polyphenols undergo less ruminal proteolysis to provide up to threefold more plant protein for intestinal digestion than alfalfa (Broderick and Albrecht, 1997; Broderick et al., 2004). While excessive levels are detrimental (Min et al., 2003), moderate levels of CT (~25 g kg⁻¹ dry matter [DM]) in fresh birdsfoot trefoil (Lotus corniculatus L.) can shift protein digestion to the intestine (Waghorn et al., 1987), increasing milk production and growth of ruminant livestock (Min et al., 1998; Woodward et al., 1999). Comparable levels of CT in ensiled birdsfoot trefoil can also enhance protein utilization and milk production of dairy cattle while mixed results were observed with ensiled red clover (Trifolium pratense L.) containing o-quinones (Broderick et al., 2001; Hymes–Fecht et al., 2005).

In addition to other benefits (Hintz et al., 1999), increased severity of conditioning at cutting can shift protein fractions in alfalfa from rapidly to slowly degraded forms (Agbossamey et al., 1998). Although not reported, severe conditioning by mechanical maceration could enhance the release of CT from vacuoles in specialized cells (Lees et al., 1995) and their interaction with plant proteases and protein substrates. Similarly, mechanical maceration could facilitate the interaction of proteins and proteases with o-quinones formed by the action of chloro-plastic polyphenol oxidase on o-diphenols stored in vacuoles (Sullivan et al., 2004).

Conservation methods can also influence proteolysis in forages. Following conservation of alfalfa, NPN as a proportion of protein is typically 25% in hay compared to 50% or more in silage (Hristov and Sandev, 1998; Kohn and Allen, 1995). Switching from silage to hay conservation can also reduce rumen proteolysis and enhance microbial protein synthesis to improve animal production (Peltekova and Broderick, 1996; Vagnoni and Broderick, 1997). By contrast, the effect of hay vs. silage conservation on protein fractions in polyphenol-containing forages has received scant attention (Kraiem et al., 1990).

In this study, forages containing CT or o-quinones or lacking these polyphenols were conventionally conditioned or macerated to vary the degree of cell breakage and the potential for polyphenol–protein interactions during conservation as hay or silage. Hays and silages were then treated with buffer, protease, and acid-detergent solutions to assess how forage polyphenols and methods of conditioning and conservation affect protein fractions and the potential performance of livestock.

**MATERIALS AND METHODS**

**Forage Production**

Base, low, and high CT populations of ‘NC-83’ birdsfoot trefoil (Miller and Ehlie, 1996; ‘Cinnamon’ or ‘Marathon’ red clover, and a highly dormant alfalfa (‘ZG9910’) derived from Spredor 3 and Pioneer 5151 (J. Moutray, personal communication, 2002) were conventionally seeded on 10 Aug. 2001 and 18 Apr. 2002 near Prairie du Sac, WI. The Richwood silt loam soil (fine-silty, mixed, superactive, mesic Typic Argiudoll) at the site had very high levels of available P (74 kg ha⁻¹) and exchangeable K (292 kg ha⁻¹), adequate levels of B and S, and a pH of 7.1. The year following seeding, spring growth was harvested between 13 June and 20 June in 2002 and between 29 May and 2 June in 2003. Summer regrowth was harvested about 40 d after the spring cuttings. In 2002, harvest of spring growth was delayed until slowly maturing birdsfoot trefoil reached late bud stage while red clover and alfalfa were at 10 to 50% flowering. Summer regrowth in 2002 was harvested at 10 to 50% flowering. In 2003, forages were harvested at late bud for spring growth and at 10% flowering for summer regrowth. Between late April and harvest of spring growth, average temperatures were similar both years (12°C), but precipitation was greater in 2002 (26 cm) than in 2003 (11 cm). Summer regrowth in 2002 occurred under warmer and drier conditions (22°C average temperature, 6.5 cm precipitation) than in 2003 (18°C average temperature, 13 cm precipitation).

At each harvest, plots were cut near midday at a 5-cm height and weeds were removed by hand. A subsample of herbage was frozen in liquid N and subsequently freeze-dried for analysis of CT. Fresh herbage was then conditioned (Fig. 1) by passage through intermeshing rubber rolls set at manufacturer specifications (New Holland, New Holland, PA) or by rotary-impact maceration (Kraus et al., 1999). Conditioned herbage was then wilted on plastic mesh screens (1-mm openings) in forced-draft ovens run at 35°C from 0900 to 1800 h; ovens were turned off at night. After drying for about 24 h to a DM content of 350 g kg⁻¹, macerated herbage (700 g) and coarsely chopped roll-conditioned herbage (500 g) were ensiled in 1-L glass canning jars. Herbage remaining on screens was oven-dried at 35°C as hay. After incubating at room temperature for 90 d, silages were removed from jars, frozen in liquid N, and freeze-dried. Dried hay and silage samples were ground through a cyclone mill (2-mm screen) for analysis.

**Forage Analyses**

Laboratory assays were run in duplicate. Cell breakage in roll-conditioned, chopped, and macerated herbage wilted to 35% DM was estimated by a conductivity index method (Kraus et al., 1999). Ground samples were analyzed for DM by drying.
were suspended and pelleted (4500 × g, 5 min) thrice with 35 mL of ice-cold water and then transferred with water to glassfiber filters. Residues and filters were analyzed by combustion to estimate RDP, with correction for filter N. Insoluble RDP (IRDP) was calculated as RDP minus SP. Subtracting RDP from CP provided an estimate of rumen-undegradable protein (RUP) flowing to the gastrointestinal tract. Finally, the quantity of intestinal available protein (IAP) was estimated as RUP minus “indigestible” acid-detergent insoluble protein (Auffere and Guerin, 1996), which was determined on samples (0.5 g) sequentially extracted in filterbags with neutral detergent (run without amylase, sodium sulfate, acetone washing, or oven drying) followed by acid detergent using a fiber analyzer (ANKOM Technology, Macedon, NY). Acid detergent residues and filter bags were analyzed by combustion to estimate acid-detergent insoluble protein, with correction for filter bag N.

**Statistical Design and Analyses**

The experimental design was a randomized complete block with four field replications and a split-split plot arrangement of treatments. Forage entry was the main plot, conditioning level was the subplot, and conservation method was the sub-subplot. Data were analyzed using PROC MIXED (SAS Institute, 2003). Forage genotype, conditioning level, conservation methods, harvests, and years were fixed effects. Blocks within years and associated interactions were random effects. Pairwise comparisons of least square means were performed when a significant F-test was detected at P ≤ 0.05. Differences mentioned in the text were significant at P ≤ 0.05.

The responses of protein fractions in birdsfoot trefoil to CT were tested by regression analyses (Neter et al., 1990; SAS Institute, 2003). A reduced model (i.e., roll conditioned and macerated birdsfoot trefoil had the same slope and intercept) was rejected if it significantly increased error sums of squares (P ≤ 0.05) compared to the full model (i.e., roll conditioned and macerated birdsfoot trefoil had unique slopes and/or intercepts). If the reduced model was rejected, then an indicator variable was added to the equation to test whether the intercept and/or slope of roll-conditioned trefoil differed from macerated trefoil at P ≤ 0.05. Similar approaches were used to test whether protein fraction responses to CT differed between red clover and birdsfoot trefoil.

**RESULTS**

**Herbage Polyphenols**

To help isolate CT effects on protein fractions, we used three populations of birdsfoot trefoil differing in CT expression. Concentrations of CT in freeze-dried herbage increased threefold from the low to the high CT population (Table 1). Averaged across populations, CT levels were 42% greater in 2002 than in 2003 and 88% greater in summer regrowth than in spring growth. Year and harvest effects were, however, most pronounced for the high CT population. CT was a minor component in red clover (<3 g kg⁻¹ DM) and absent in alfalfa.

The main polyphenols in red clover, comprising ~20 g kg⁻¹ DM, are o-diphenols (e.g., phasic acid and clovamide)
which are converted into protein-binding \( \alpha \)-quinones by polyphenol oxidase (Sullivan and Hatfield, 2006). At the onset of this work, genotypic variation in polyphenol oxidase or \( \alpha \)-diphenol expression had not been reported for red clover so only one variety was evaluated each year. Unfortunately, quantification of \( \alpha \)-diphenols by chromatographic methods is hindered by a lack of appropriate standards (Sullivan and Hatfield, 2006). Colorimetric methods for polyphenols, such as the Prussian blue assay (Schofield et al., 2001), also lack sufficient specificity for \( \alpha \)-diphenol substrates of polyphenol oxidase (J.H. Grabber, unpublished data, 2006). Therefore, no attempt was made to quantify \( \alpha \)-diphenol levels in red clover. Soluble \( \alpha \)-diphenols are present in birdfoot trefoil (e.g., CT precursors such as epicatechin) but absent in alfalfa; both species, however, lack polyphenol oxidase activity (Jones et al., 1995a; Sullivan and Hatfield, 2006).

Conditioning and Cell Breakage

A leachate conductivity index was used to estimate cell breakage in roll-conditioned, chopped, and macerated herbage relative to homogenization with a Waring blender, which breaks \( \sim \)85% of the cells in alfalfa (Kraus et al., 1999). The conductivity index of roll-conditioned herbage was very low (0.06), with no differences noted between forage species. Chopping before ensiling increased cell breakage by about threefold, with the conductivity index of alfalfa (0.19) and red clover (0.23) being greater than that of birdsfoot trefoil (0.16). Mechanical maceration substantially increased cell breakage, but the conductivity index of alfalfa and red clover (0.84) remained somewhat greater than that of birdsfoot trefoil (0.79). Harvest or year had little or no impact on cell breakage. Overall, shifting from roll conditioning to maceration increased cell breakage by about 13-fold in hay and by fourfold in forage chopped for ensiling. Although not measured, differences in cell breakage between conventionally processed and macerated forage should diminish during ensiling because anaerobic conditions promote cell lysis.

Crude Protein

On a DM basis, the average CP content in hay (203 g kg\(^{-1}\)) was slightly lower than in silage (210 g kg\(^{-1}\)). The effects of forage genotype and conditioning method were usually small (Table 2), but harvest and year influenced CP concentrations. The average CP content of hay and silage in spring growth (167 g kg\(^{-1}\)) and summer regrowth (197 g kg\(^{-1}\)) from 2002 were lower than in spring growth (227 g kg\(^{-1}\)) and summer regrowth (236 g kg\(^{-1}\)) from 2003. A later spring harvest and warmer summer temperatures probably contributed to lower CP levels in 2002. Other effects of harvest, year, and their associated interactions on CP levels were small or not significant.

<table>
<thead>
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<th>Year and harvest</th>
<th>Alfalfa</th>
<th>LT trefoil</th>
<th>BT trefoil</th>
<th>HT trefoil</th>
<th>Red clover</th>
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<td>14.8a</td>
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</tbody>
</table>

\(^1\)ND, none detected.

\(^2\)Means within rows with unlike letters differ (\( P < 0.05 \)).

**Crude Protein Fractions**

**Soluble Protein**

Plant proteases convert most SP in hays and essentially all SP in silages to NPN (Hristov and Sandev, 1998; Kohn and Allen, 1995), thus SP is a useful indicator of plant protease action during forage conservation. On a CP basis, SP in roll-conditioned alfalfa averaged 440 g kg\(^{-1}\) for hay compared to 734 g kg\(^{-1}\) for silage where prolonged hydration and anaerobic lysis promoted proteolysis. In roll-conditioned hays and silages of birdsfoot trefoil, SP declined from the low to high CT populations, with the latter containing \( \sim \)70 g kg\(^{-1}\) less SP than alfalfa (Table 2). The apparent effect of \( \alpha \)-quinones in roll-conditioned red clover was more dramatic, reducing SP by 107 g kg\(^{-1}\) in hay and 219 g kg\(^{-1}\) in silage compared to alfalfa.

Shifting from roll conditioning to maceration of herbage lowered SP by an average of 115 g kg\(^{-1}\) in hays and 171 g kg\(^{-1}\) in silages (Table 2). Although maceration greatly accentuated browning of red clover tissue, reductions in SP for red clover and alfalfa were similar, averaging 123 g kg\(^{-1}\) for hay and 146 g kg\(^{-1}\) for silage. In birdsfoot trefoil hays, reductions in SP due to maceration ranged from 99 g kg\(^{-1}\) in the low CT population to 127 g kg\(^{-1}\) in the high CT population. Maceration effects were greater in birdsfoot trefoil silages, but SP declined by an average of 188 g kg\(^{-1}\) in all populations, regardless of CT levels.

Harvest did not influence SP levels in roll-conditioned hays and silages. But in hays, shifting from roll conditioning to maceration reduced SP by 97 g kg\(^{-1}\) in spring growth compared to 134 g kg\(^{-1}\) in summer regrowth. Conversely in silage, maceration of spring growth reduced SP by 148 g kg\(^{-1}\) in birdsfoot trefoil compared to 128 g kg\(^{-1}\) in alfalfa and red clover, while maceration of summer regrowth reduced SP by 227 g kg\(^{-1}\) in birdsfoot trefoil compared to 164 g kg\(^{-1}\) in alfalfa and red clover. Harvest did not affect SP in 2002, but SP in spring growth exceeded that in summer regrowth by 46 g kg\(^{-1}\) for hays and 81 g kg\(^{-1}\) for silages in 2003. Other interactions involving year or harvest were not
Table 2. Concentrations of crude protein (CP), soluble protein (SP), insoluble rumen-degradable protein (IRDP), rumen-undegradable protein (RUP), and intestinal available protein (IAP) fractions in forage legume hays and silages. Alfalfa, low tannin (LT), base tannin (BT) and high tannin (HT) birdsfoot trefoil populations, and red clover were conditioned with rolls or macerated immediately after harvesting. Data are averaged over 2 yr and two harvests per year.

<table>
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<th>Fraction</th>
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<th>BT trefoil</th>
<th>HT trefoil</th>
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<td>206ab</td>
<td>201b</td>
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<td>197b</td>
<td>194b</td>
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</tr>
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*R means within rows with unlike letters differ (P < 0.05).
† Conditioning treatment means with unlike letters differ (P < 0.05).

significant or small in magnitude. Across all harvests over both years, SP was negatively associated with CT levels in birdsfoot trefoil (Fig. 2).

**Insoluble Rumen Degradable Protein**

Insoluble rumen-degradable proteins are of interest because they are used more efficiently than SP for microbial protein synthesis (Aufrere and Guerin, 1996). On a CP basis, IRDP in roll-conditioned alfalfa averaged 305 g kg⁻¹ for hay compared to 67 g kg⁻¹ for silage where plant proteases extensively degraded proteins during ensiling. Relative to alfalfa, IRDP in roll-conditioned birdsfoot trefoil hays declined slightly from the low to the high CT populations, whereas o-quinones apparently had no effect on IRDP in red clover (Table 2). In roll-conditioned silages, birdsfoot trefoil populations had similar IRDP levels, which were slightly lower than alfalfa, while red clover had IRDP levels twofold greater than alfalfa (Table 2). Changing from roll-conditioning to maceration had similar effects in hays and silages, increasing IRDP by an average of 103 g kg⁻¹ in alfalfa, 179 g kg⁻¹ in red clover, and 50 g kg⁻¹ in all birdsfoot trefoil populations.

In hays, IRDP with roll conditioning was 24 g kg⁻¹ greater in summer regrowth than in spring growth, while the response to maceration was 50 g kg⁻¹ greater in summer regrowth than in spring growth for alfalfa and red clover with no harvest effects for birdsfoot trefoil. With roll conditioning, hay IRDP for both harvests in 2002 were 61 g kg⁻¹ less than in 2003 while the response to maceration was only 42 g kg⁻¹ for spring growth in 2003 compared to 107 g kg⁻¹ for all other harvests. In silages, both harvests had similar IRDP with roll conditioning, but summer regrowth had a 31 g kg⁻¹ greater response to maceration than spring growth. Year did not influence silage IRDP levels in birdsfoot trefoil populations (regardless of conditioning method) or in roll-conditioned alfalfa silage, but the response of alfalfa to maceration was 63 g kg⁻¹ greater in 2002 than in 2003. By contrast, silage IRPD in red clover was 58 g kg⁻¹ greater in 2002 than in 2003 with roll conditioning while the response to maceration was 75 g kg⁻¹ less in 2002 than in 2003. Other interactions with harvest or year were small in magnitude or not significant.

**Rumen-Undegradable Protein and Intestinal Available Protein**

Due to excessive proteolysis, an inadequate level of alfalfa RUP usually escapes the rumen for digestion in the gastrointestinal tract. Subtracting acid-detergent insoluble protein from RUP gives an estimate of IAP that is actually utilized by livestock (Aufrere and Guerin, 1996). Acid-detergent insoluble protein levels averaged only 38 g kg⁻¹ CP and were fairly consistent across treatments (data not shown). As a result, IAP levels were slightly lower but parallel to RUP. In roll-conditioned alfalfa, RUP on a CP basis averaged 255 g kg⁻¹ for hay and 199 g kg⁻¹ for silage while IAP averaged 221 g kg⁻¹ for hay and 165 g kg⁻¹ for silage. Among roll-conditioned hays and silages of birdsfoot trefoil, RUP and IAP levels increased from the low to high CT populations, with the latter containing ~95 g kg⁻¹ more RUP and IAP
than alfalfa (Table 2). Presumably due to o-quinone protection of protein, RUP and IAP concentrations in roll-conditioned red clover exceeded alfalfa by 110 g kg\(^{-1}\) for hay and 144 g kg\(^{-1}\) for silage. Switching from roll conditioning to maceration increased RUP and IAP in alfalfa by only 15 g kg\(^{-1}\) in hay and 44 g kg\(^{-1}\) in silage. Although they differed in CT content, birdsfoot trefoil populations responded similarly to maceration, with RUP and IAP gains of 53 g kg\(^{-1}\) in hay and 138 g kg\(^{-1}\) in silage. Surprisingly after maceration, birdsfoot trefoil silage had more RUP and IAP than hay. In red clover, shifting from roll conditioning to maceration unexpectedly reduced RUP and IAP by 65 g kg\(^{-1}\) in hay and 28 g kg\(^{-1}\) in silage.

In birdsfoot trefoil and red clover but not alfalfa, hay RUP was 48 g kg\(^{-1}\) greater in summer regrowth than in spring growth and 56 g kg\(^{-1}\) greater in 2002 than in 2003.

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Figure 2. Forage crude protein (CP) fractions (soluble protein, rumen-undegradable protein, and intestinal available protein) in hays and silages from two cuttings in 2002 and 2003 as influenced by condensed tannins and conditioning method. Regressions were fitted to birdsfoot trefoil data and were significant at \(P < 0.05\).
A similar interaction of forage genotype with harvest was observed for hay IAP. For silage, both harvests had similar RUP in 2002, but in 2003 spring growth had 57 g kg$^{-1}$ less RUP than summer regrowth. Shifting from roll conditioning to maceration of birdsfoot trefoil silage yielded a 48 g kg$^{-1}$ greater response in RUP and IAP for summer regrowth than for spring growth but no harvest response was observed for alfalfa and red clover. Shifting from roll conditioning to maceration of alfalfa silage increased RUP and IAP by 28 g kg$^{-1}$ in 2002 and 59 g kg$^{-1}$ in 2003, but maceration of red clover silage reduced RUP and IAP by 52 g kg$^{-1}$ in 2003 with no effect in 2002. In contrast for birdsfoot trefoil, maceration increased silage RUP and IAP by 98 to 169 g kg$^{-1}$, but the response of each population did not follow a consistent pattern across years. Other interactions with harvest and year for RUP and IAP in hay and silage were small in magnitude or not significant. Across all harvests and years, RUP and IAP were positively associated with CT levels in birdsfoot trefoil (Fig. 2).

**DISCUSSION**

**Polyphenol, Conditioning, and Conservation Effects on Protein Fractions**

Overall, levels of SP, IRDP, RUP, and IAP were affected much more by conservation method, forage genotype, and conditioning method than by harvest or year. Among the factors examined, conserving forages as hay rather than silage usually had the greatest impact on decreasing SP and increasing IRDP levels (Table 2). In contrast, RUP and IAP were frequently influenced more by forage genotype than conditioning or conservation methods.

Amid the forages examined, polyphenol-free alfalfa generally had the highest levels of SP, intermediate to high levels of IRDP, and the lowest levels of RUP and IAP (Table 2). Shifting from roll conditioning to maceration dramatically reduced SP and increased IRDP levels in alfalfa with little effect on RUP and IAP, suggesting that maceration of alfalfa mainly facilitated protein avoidance of plant protease action. The actual mechanisms responsible for reduced SP in macerated alfalfa are not known, but enhanced tissue disruption could facilitate coprecipitation of proteins with cellular constituents or prevent up-regulation of plant proteases during forage conservation (Kingston-Smith et al., 2003). Although maceration could increase protein exposure to proteases stored in plant organelles, other factors apparently prevailed to depress overall plant proteolysis in alfalfa.

As noted previously for freeze-dried herbage or direct cut silage (Broderick and Albrecht, 1997; Albrecht and Muck, 1991), CT in birdsfoot trefoil decreased SP and increased RUP in roll-conditioned hays and wilted silages. Condensed tannins are thought to limit proteolysis by binding and precipitating plant proteases and protein substrates (Min et al., 2003). As illustrated in Fig. 2, CT levels accounted for 67 to 80% of the variation in SP, RUP, and IAP in roll-conditioned birdsfoot trefoil hays. In these hays, SP declined by 1.9 g g$^{-1}$ of CT while RUP and IAP increased by 1.25 g g$^{-1}$ of CT, but the response of SP diminished as CT levels increased. In roll-conditioned birdsfoot trefoil silages, CT accounted for ~60% of the variation in protein fractions; SP declined and RUP and IAP increased by 1.4 g g$^{-1}$ of CT. In vitro estimates of legume protein precipitation by isolated *Lotus* CT vary from 0.9 to 12.5 g g$^{-1}$ (McAllister et al., 2005; McNabb et al., 1998; Perez-Maldonado et al., 1995). The current SP data suggest the lower in vitro values may best reflect protein precipitation within tissues.

Levels of SP, RUP, and IAP were more highly related to CT concentrations in macerated birdsfoot trefoil, accounting for 80 to 96% of the variation in hays and about 82% of the variation in silages (Fig. 2). Thus, switching from roll conditioning to maceration improved the uniformity of CT action on protein. Shifts in SP, IRDP, RUP, and IAP due to maceration were not consistently related to harvest or yearly variations in CT levels. As a result, maceration shifted intercepts ($P < 0.05$) of SP, RUP, and IAP with little effect on regression slopes, indicating that maceration altered protein fractions by mechanisms largely independent of CT. Consequently, membrane lysis during conventional forage conservation evidently permits adequate interaction and protection of proteins by CT. Since reductions in SP due to CT led to corresponding increases in RUP, it is apparent that proteins protected from plant proteases during conservation also avoided degradation by *Streptomyces* proteases in fully cured hay and silage. Thus in both roll-conditioned and macerated hays and silages of birdsfoot trefoil, CT mainly acts by protecting protein substrates from protease action.

Recent work (Sullivan and Hatfield, 2006) has clearly demonstrated that $o$-quinones are the main factor limiting proteolysis in red clover. The mechanism of $o$-quinone action has not been clearly delineated, but it may involve coupling and direct inactivation of plant proteases or cross-linking and protection of protein substrates from protease action. Data from this study suggest that the mechanisms of proteolytic inhibition in red clover varied with the conditioning and conservation methods employed. For roll-conditioned hays, red clover had ~110 g kg$^{-1}$ less SP and ~110 g kg$^{-1}$ more RUP than alfalfa and birdsfoot trefoil (at comparable CT levels, Fig. 2), strongly implicating $o$-quinones as the main factor limiting proteolysis in red clover. Since SP decreased and RUP increased concurrently in roll-conditioned red clover hay, $o$-quinones probably acted mainly by cross-linking and protecting protein substrates from both plant and *Streptomyces* proteases. In the case of roll-conditioned silage, red clover contained 219 g kg$^{-1}$ less SP than alfalfa with two-thirds (143 g kg$^{-1}$) presumably
being partitioned into RUP by o-quinone protection of protein substrates. The remaining one-third (77 g kg\(^{-1}\)) contributed to the IRDP fraction, probably having escaped proteolysis during ensiling due to o-quinone inactivation of plant proteases. In a similar manner, roll-conditioned silage of red clover also had \(~240\) g kg\(^{-1}\) less SP and \(~140\) g kg\(^{-1}\) more RUP than birdsfoot trefoil at similar CT levels, again implicating o-quinone protection of protein substrates as being more important than o-quinone inactivation of plant proteases for limiting proteolysis. Direct inactivation of Streptomyces proteases in the RDP assay by o-quinones would be unlikely because polyphenol oxidase activity and o-diphenol substrates would probably be lost during hay curing or silage fermentation.

Although switching from roll conditioning to maceration caused comparable reductions in SP for red clover, alfalfa, and birdsfoot trefoil, maceration surprisingly caused a net reduction in RUP only for red clover. Interestingly, RUP in macerated hay and silage of red clover responded identically to birdsfoot trefoil to variations in CT (Fig. 2), suggesting that maceration completely disrupted o-quinone protection of protein substrates, leaving CT as the only polyphenol protecting proteins from Streptomyces proteases. Exactly how maceration alters o-quinone action is not known but accelerated oxidation of o-diphenols could alter coupling reactions of o-quinones, analogous to what occurs with lignin precursors (Sarkanen, 1971). For example, gradual o-diphenol oxidation by polyphenol oxidase in roll-conditioned herbage could favor nucleophilic coupling of o-quinones to proteins followed by oxidative cross-linking of o-diphenol-protein complexes. Such cross-linking would protect proteins from both plant and Streptomyces protease action. By contrast, rapid oxidation of o-diphenols by polyphenol oxidase with maceration may favor the initial coupling of o-quinones to proteins at the expense of continued coupling reactions to cross-link o-diphenol–protein complexes. Under this scenario, nucleophilic attack of o-quinones would inactivate plant proteases to preserve protein during forage conservation but a lack of cross-linking would leave protein substrates vulnerable to subsequent proteolysis by Streptomyces proteases (and presumably ruminal proteases as well). In any case, researchers should consider the potential effects of maceration on o-quinone–protein interactions as they seek to elucidate the physiological role and mechanism of action of polyphenol oxidase-generated o-quinones.

Forage Protein Fractions and Dairy Cattle Requirements

Lactating dairy cattle have relatively high protein requirements and would benefit from increases in forage RUP and IAP. When fed high-forage diets with adequate CP, large breed dairy cattle require about 350 g RUP kg\(^{-1}\) CP to produce 35 kg d\(^{-1}\) of milk (National Research Council, 2001). Although diets will include other ingredients to avoid nutrient deficiencies, this RUP level represents a benchmark for improving forage protein utilization by dairy cattle. While hay and particularly silage of alfalfa falls short, roll-conditioned birdsfoot trefoil could reach this benchmark with CT levels of \(~70\) g kg\(^{-1}\) CP in hay and \(~120\) g kg\(^{-1}\) CP in silage (Fig. 2). Based on these results, expression of comparable levels of CT in roll-conditioned alfalfa could boost RUP to acceptable levels. With maceration, CT levels as low as 30 g kg\(^{-1}\) CP might be sufficient to meet the RUP target in birdsfoot trefoil silage and hay. Although less responsive than birdsfoot trefoil, maceration of CT-containing alfalfa could further boost RUP to acceptable levels, particularly if CT expression is marginal. Expression of higher CT levels could further heighten RUP and milk production, provided that IAP is not depressed as has been observed for many high CT forages (Min et al., 2003). Since low levels of acid-detergent insoluble protein in all birdsfoot trefoil populations were observed, moderate expression of CT in alfalfa should also boost IAP levels. This is supported by feeding trials with birdsfoot trefoil where CT increased intestinal absorption of essential amino acids in sheep by 60%, and milk production in dairy cattle by 2.5 to 4.9 kg d\(^{-1}\) (Hymes-Fecht et al., 2005; Waghrorn et al., 1987; Woodward et al., 1999). While acid-detergent insoluble protein is generally accepted as a good predictor of indigestible protein in CT-free forages, the nutritional relevance of this assay for various types of CT-containing forages requires further validation by in situ or in vivo animal trials. Regardless of the conditioning or conservation method, frequent and accurate estimates of RUP and IAP will be needed to optimize animal production because, as noted above and elsewhere (Cassida et al., 2000; Miller and Ehlik, 1996), CT expression varies depending on plant maturity and growth environment.

In the case of red clover, these assays indicate the before-mentioned RUP target of 350 g kg\(^{-1}\) CP could be met if hay or silage were roll-conditioned rather than macerated. Red clover had low levels of acid-detergent insoluble protein, indicating RUP in should have high intestinal availability. Despite a higher predicted RUP than alfalfa, milk production of dairy cattle fed red clover has been below expectations, being comparable to or lower than alfalfa-based diets (Broderick et al., 2001). This suggests red clover has poor intestinal availability of RUP or deficiencies of specific amino acids coupled to o-quinones that are not reflected in assays of acid-detergent insoluble protein. Clearly, additional studies are needed to properly characterize the ruminal degradability and intestinal availability of various o-quinone–protein complexes. Such work may identify strategies for optimizing polyphenol oxidase and o-diphenol expression in red clover and alfalfa to increase both the concentration and intestinal availability of RUP. Finally, since modest shifts from SP to IRDP
enhance microbial protein synthesis and milk production with alfalfa (Peltekova and Broderick, 1996; Vagnoni and Broderick, 1997), the impact of extremely high IRDP levels, particularly in macerated red clover, would be worthy of further investigation.

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