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Invited Review

The role of condensed tannins in ruminant animal production: advances, limitations and future directions

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ABSTRACT - Tannins represent one of the most abundant polyphenolic compounds in plants. Tannins exist as a multitude of chemically unique entities in nature. The most commonly occurring tannins are typically divided into two major classes based on chemical structure: hydrolysable (HT) or condensed tannins (CT). Hydrolysable tannins are esters of gallic or ellagic acid linked to a polyol core, typically glucose. Condensed tannins or proanthocyanidins consist of flavan-3-ol subunits linked together to form oligomers and polymers. Both HT and CT are defined as astringent, medium-to-high-molecular weight polyphenolic compounds that characteristically bind and precipitate soluble proteins. The objective of this paper was to present recent advances in CT-ruminant interactions, the limitations associated with understanding and using CT in ruminant animal production, and future needs for research to further advance our knowledge of the role of CT in optimization of ruminant animal production. Condensed tannins pose some anti-nutritional problems to ruminants due to their astringent property that reduces feed intake and, consequently, animal performance. Ruminants can, however, tolerate CT by slowly adapting the ruminal microbes to the toxic effects of CT and by releasing CT-binding salivary proteins. The protein-binding ability of CT has some benefits to the ruminant due to complexes formed with essential amino acids, preventing their degradation in the rumen, but releasing them in the lower gut for absorption by the animal. Recent data have suggested increased N retention when CT is given to growing animals. There are potential benefits of using CT and HT for anthelmintic purposes due to their ability to inhibit egg hatching and larval motility of gastrointestinal nematode parasites, especially in small ruminants. Condensed tannins also bind to minerals (Al, Ca, Co, Cu, Fe, Mg, Mn, P, and Zn). Although studies with ruminants have been contradictory, it has been reported that because the CT-metal ion complex is stable over a wide pH range, CT may reduce the bioavailability of minerals. Methane mitigation by feeding CT might be the most impactful benefit for ruminant production. Many empirical equations have been developed to predict ruminal methane emissions, but very few have included CT. Future research should focus on the improvement of methodology to assess CT biological activity, interaction with other plant-specialized metabolites, and associated physiological and nutritional impacts on ruminants.

Key Words: anthelmintic, bypass protein, hydrolysable tannin, methane, polyphenol, proanthocyanidin

Introduction

Tannins are a subclass of plant polyphenols, which are distinguished from other polyphenols by their ability to form complexes with and precipitate proteins (Hagerman and Butler, 1978; Hagerman, 2012). The commonly occurring tannins are traditionally broadly divided into two categories: hydrolysable (HT) and condensed tannins (CT), whose structures are distinctly different. Although tannins have been characterized predominately on the basis of their ability to bind proteins, reports dating back to as early as the 1930s describe tannins on the basis of "their astringent taste and for their many precipitation reactions with lime, lead acetate, alkaloids, gelatin, albumin, and other proteins, and also for their color reactions with iron salts" (Maitland et al., 1936). Many of these interactions with tannins and organic compounds or trace elements are still of interest today in agricultural research, especially where their potential impact on animal production is concerned. This is likely due to the many challenges and limitations associated with definitively answering questions related to the interactions of tannins in animal physiology and nutrition. It has become evident

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that furthering our understanding of using tannins in animal production is going to require a better understanding of the role of tannin chemistry in animal interactions. Perhaps this was evident in the 1920s, when Freudenberg put forth a classification scheme for tannins including HT, CT, and an unclassified group of tannins (Maitland et al., 1936), the latter suggesting the existence of tannin structures that were not well understood.

Chemical diversity of tannins

Hydrolysable tannins are typically comprised of a polyol core molecule, usually glucose, but other core molecules can include glucitol, hammamelose, shikimic acid, quinic acid, and quercitol (Hagerman, 2011), whose hydroxyl group of the core polyols have been esterified with gallic acid. Although assembled from relatively simple components, the complexity of HT increases through addition of subsequent galloyl groups, intramolecular oxidative coupling of the galloyl groups of the substituted polyol core, ring opening of glucose core, and oligomerization of the resulting entities through intermolecular oxidative coupling. Based on the structural features arising from these chemical transformations, HT can be divided into three major subclasses: galloglucoses, gallotannins, and ellagitannins. Galloglucoses are glucose molecules in which at least one glucose hydroxyl group has been esterified with gallic acid. A common representative of this subclass is 1,2,3,4,6-pentagalloglucose (PGG) (Figure 1). Gallic acid units can be added to the existing

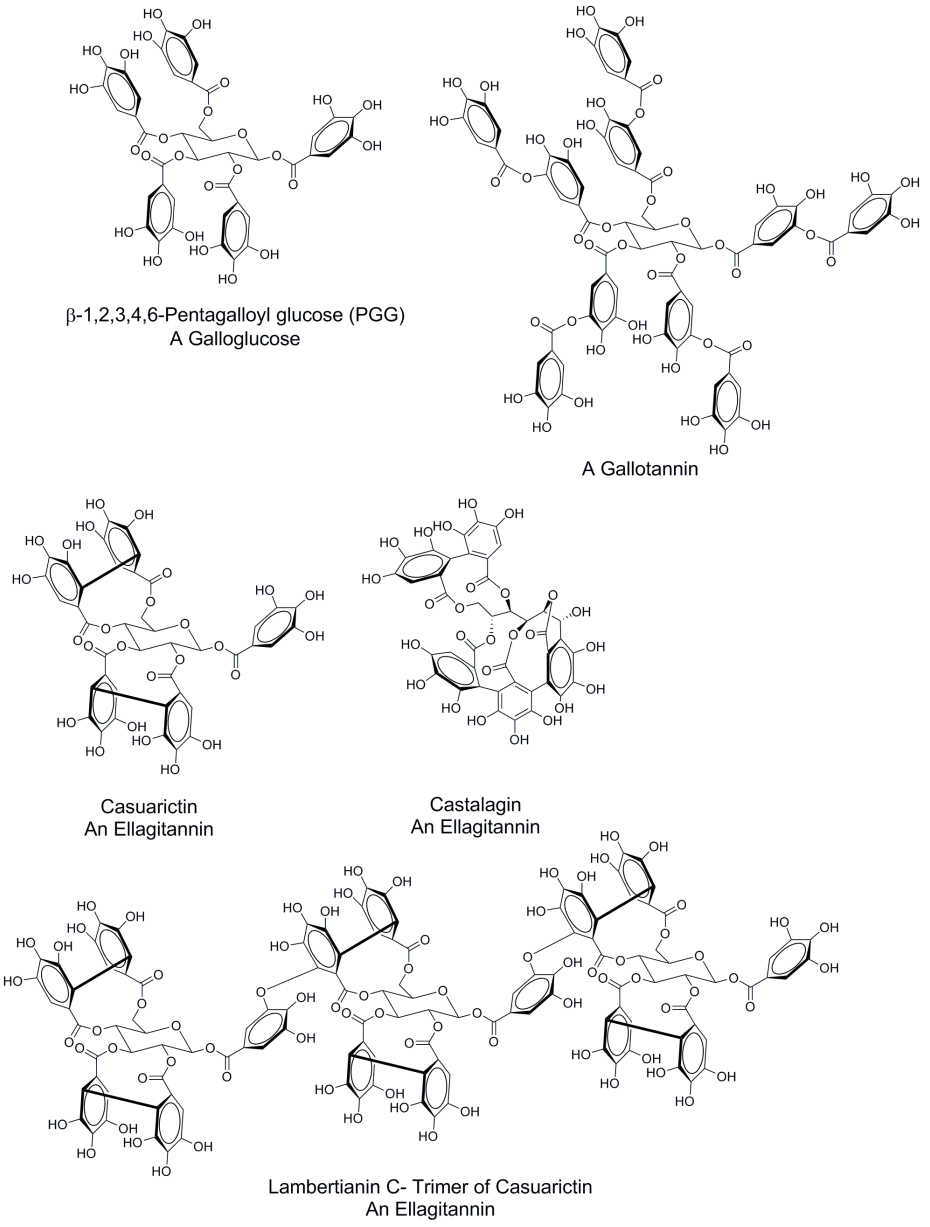


Figure 1 - Examples of hydrolysable tannins with subclasses listed.

galloyl groups of galloglucoses, which gives rise to the gallotannins. The classic example of a gallotannin is tannic acid (Figure 1). Intramolecular oxidative coupling dimerizes gallic acid substituents forming ellagic acid moieties. This coupling can occur between adjacent gallic acid such as for the conversion of PGG to tellimagrandin II through oxidative coupling of galloyl ester functionalities on the glucose C-4 and C-6 positions of PGG (Figure 1). In turn, casuarictin can be formed from tellimagrandin II through oxidative coupling of galloyl ester functionalities on C-2 and C-3. Opening of the glucose ring followed by additional oxidative couplings give rise to castalagin. These oxidative couplings can also take place intermolecularly, such as with the formation of lambertianin C, a trimer of casuarictin, through coupling of the galloyl groups at C-1 of the glucose core with the C-4/C-6 ellagic acid substituent.

Condensed tannins, also referred to as proanthocyanidins, consist of oligomers or polymers of flavan-3-ol subunits. The flavan-3-ol subunits found in CT varies, but commonly occurring ones include catechin, epicatechin, gallocatechin, and epigallocatechin (Figure 2). Flavan-3-ols differ in their hydroxyl substitution patterns and the relative stereochemistry of the C-2 and C-3 C-ring substituents (circled in Figure 2). Catechin and epicatechin have the same hydroxylation pattern (phenolic substituents on C-5 and C-7 of the A-ring, C-3 and C-4 of the B-ring, and an alcohol substituent in the C-3 carbon of the C-ring). The only difference is the relative orientation in space of the substituent attached to the C-3 of the C-ring. Regarding this relationship, if the C-2/C-3 substituents of the C-ring are represented by both dashed bonds or by both wedged bonds, the substituents are in the *cis* configuration. If one bond is represented as a dashed bond and one represented as a wedged bond, the substituents are in the *trans* configuration. When there are two or more chiral (asymmetric) centers, as dashed and wedged bonds (Figure 2), and only one of them is inverted (changed from wedge to dash or vice versa) from a pair of structures which otherwise are identical, the isomer is referred to as an epimer. Thus, catechin with the inverted carbon center at C-3 of the C-ring is referred to as epicatechin. Gallocatechin and epigallocatechin differ from catechin and epicatechin by the inclusion of an additional phenol substituent on the B-ring (indicated by the arrows in Figure 2). This substitution pattern on an aromatic ring, with three phenolic substituents on adjacent carbons, is referred to as a gallo substitution. Thus, with this substitution pattern, catechin becomes gallocatechin and epicatechin becomes epigallocatechin. Catechin and epicatechin are referred to as procyanidin (PC) subunits because, upon oxidation, both give rise to cyanidin. Similarly, gallocatechin and epigallocatechin are referred to as prodelphinidin (PD) subunits because, upon oxidation, both give rise to delphinidin.

Additional flavan-3-ol subunits are present in CT from other plant materials, but occur less frequently than PC and PD subunits. These flavan-3-ol subunits differ in the number of hydroxyl (OH) groups they contain versus PC and PD subunits (Figure 2). These compounds also occur as the epimeric isomers (not shown).

The assemblage of flavan-3-ol subunits into CT oligomers and polymers can occur via several types of covalent bonding patterns (Figure 3). The most common occurring bonding patterns include covalent linkages from the C-4 position of the C-ring of one flavan-3-ol to the C-8 carbon of the subsequent flavan-3-ol subunit or the C-4 position of the C-ring of one flavan-3-ol to the C-6 carbon of the subsequent flavan-3-ol subunit. These linkages are referred to as B-type linkages and are specifically labeled

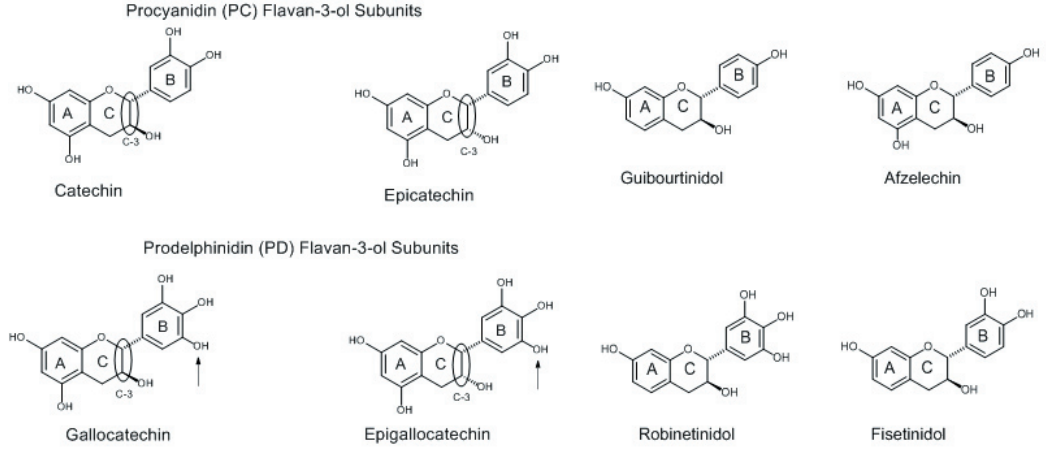


Figure 2 - Structures of flavan-3-ols occurring in condensed tannins.

4→8 or 4→6 B-type linkages. A third relatively prevalent interflavan linkage involves formation of two covalent bonds between participating flavan-3-ol subunits. For example, in addition to an existing 4→8 B-type linkage, an additional covalent bond is formed from the C-7 oxygen atom, from the carbon next to the participation C-8 carbon to the C-2 carbon of the 4→8 connected flavan-3-ol subunit, and is referred to as a 4→8, 2→O→7 bonding pattern and commonly referred to as A-type interflavan linkages. A-type linkages can be found in CT isolated from cranberries (Foo et al., 2000), peanut skins (Lou et al., 1999), and cinnamon (Anderson et al., 2004).

Hydrolysable tannins are believed to be toxic to livestock, including ruminants. Doce et al. (2013) confirmed this by feeding cattle the leaves of *Quercus pyrenaica*, a genus known to be rich in HT, and reported a marked inhibition of digestion and symptoms of acute toxicity primarily when conditions of feed restriction and malnutrition were prevalent. This may be due to greater protein binding activity of some HT as compared with some CT (Jayanegara et al., 2015). Given the fact that HT (e.g. punicalagin) have hepatotoxic and nephrotoxic effects on some livestock (Filippich et al., 1991), CT are often the focus of research associated with tannin-animal interactions. Nonetheless, future applications of HT as antiviral and antimicrobial compounds (Buzzini et al., 2008) are still a topic of interest for many ruminant nutritionists.

Not all plant species produce CT; and among those that do, concentration and chemical characteristics are highly variable. Some forage plants, such as legumes that are rich in CT, are also generally rich in nutritive value (e.g. protein). For animals that consume CT-containing legumes, perhaps the high nutritive value of the legume helps to counteract the often-observed anti-nutritional effects of CT when animals consume high concentrations of biologically active forms.

The anti-nutritional effect of condensed tannins

Although it is frequently overlooked or disregarded, the fact that much of the biologically active CT-producing plants demonstrate anti-nutritional effects on animals that consume them cannot be ignored. However, generalizations regarding anti-nutritional effects of CT on animals are common. Examples of these generalizations include decreased diet palatability, depressed intake at dietary CT concentrations exceeding 5% of dry matter (DM), depressed digestibility of nutrients (protein, carbohydrates, and fats), and depressed feed efficiency and production of animal products. Mueller-Harvey (2006) provided some much-needed clarification and context for better understanding these anti-nutritional responses.

Palatability is often based on astringency associated with CT-protein complexes formed from proteins in saliva; thus, the greater the protein bound by CT, the greater the astringency and the lower the palatability. However, not all CT bind protein equally. For example, *Desmodium paniculatum* produces a greater concentration of CT that demonstrate lower protein binding as compared with *Neptunia lutea* or *Lespedeza cuneata* that produce lower concentrations of CT with more protein-binding activity (Naumann et al., 2014b). This may explain why *Onobrychus viciifolia* containing 9-10% CT was more palatable to sheep than *Lotus corniculatus*, which contain much lower concentrations of CT at 2.6-4% (Häring et al., 2008). Intake is, at least initially, related to palatability. It is possible that

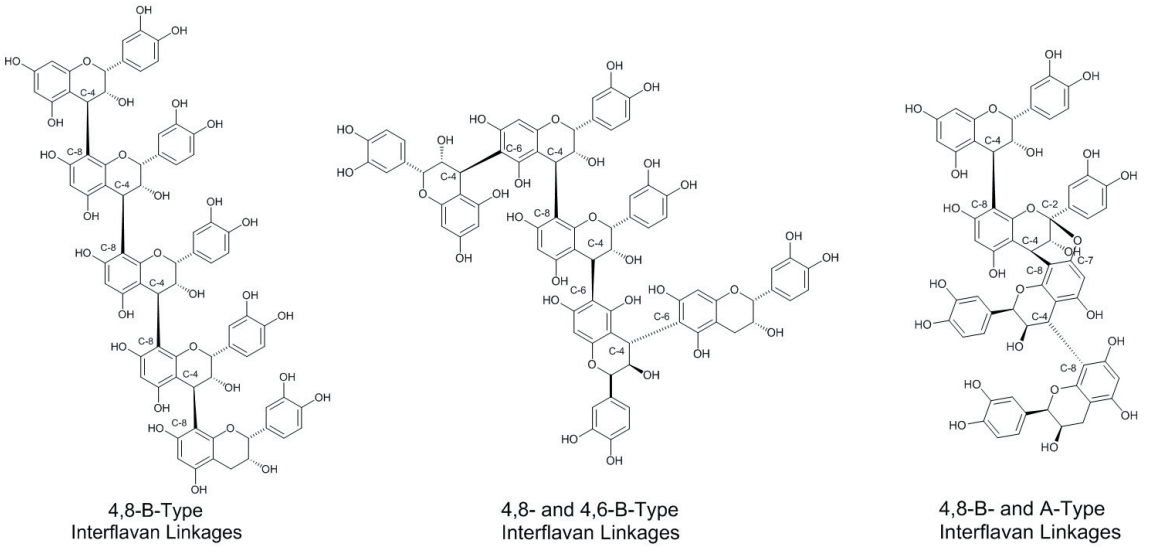


Figure 3 - Example of different types of interflavan linkages occurring in condensed tannin oligomers and polymers.

intake may be depressed at concentrations less than 5% of DM when the CT are more effective at protein binding and at concentrations greater than 5% DM when the CT are less effective.

Observed decreases in nutrient digestibility by CT are inconsistent. For example, *Dalea purpurea* with CT concentrations ranging from 6-9% DM had no negative impact on *in vivo* crude protein (CP) or DM digestibility (Jin et al., 2012). However, Jayanegara et al. (2015) observed decreases in *in vivo* organic matter (OM) digestibility when CT from different sources and with different chemical structures were added to the substrate. Theodoridou et al. (2010) observed depressed ruminal protein digestibility and a shift from urinary N excretion to fecal N excretion while maintaining body-N retention in sheep fed *O. viciifolia* containing CT at concentrations of 2.5-3.4% DM. *In vivo* or *in situ* studies such as this one mentioned above provide insight into and a greater understanding of the complexity of the relationship between CT and animal nutrition.

The fact that many of the plants that produce CT also produce other plant specialized metabolites cannot be overlooked. It is common to measure CT in the plant or diet and observe animal responses. However, there may be other metabolites that go unmeasured that are responsible for the observed response. For example, *Acacia angustissima* is a legume that produces a moderate concentration of CT (7-9% DM) and suppresses ruminal methane production (Naumann et al., 2013b), binds protein (Naumann et al., 2014b), and inhibits larval migration of L3 *Haemonchus contortus* (Naumann et al., 2014a). Condensed tannins may be the only one, of the many plant specialized metabolites found in *A. angustissima*, affecting these responses (McSweeney et al., 2008). These compounds, individually or in combination, could potentially overwhelm the ability of the animal to detoxify. However, if animals and their rumen microbial populations are allowed to adapt to *A. angustissima*, symptoms commonly associated with toxicity from this plant are dampened or avoided (Odenyo et al., 1997).

Microbial adaptation to CT is one of the key protective mechanisms for the ability of animals to avoid anti-nutritional effects. The mechanism by which microbial adaptation protects the animal is unclear, but may be related to shifts in population towards microbes that have the ability to alter the CT (Smith et al., 2005). Goats fed leaves from *Quercus semicarpifolia* and inoculated with a live culture of *Streptococcus gallolyticus*, a tannin-degrading microorganism, experienced greater DM and CP digestibility, ADG, and feed efficiency as compared with uninoculated goats (Kumar et al., 2014). A shift in rumen microbial population towards *Prevotella* spp. was observed in Sika deer (*Cervus Nippon*) fed leaves from *Quercus* spp., suggesting that either conditions changed in the rumen that resulted in promoting *Prevotella* or that *Prevotella* may be able to degrade *Quercus* tannins (Li et al., 2013). The tannins in *Quercus* spp. are generally HT rather than CT. However, *S. gallolyticus* also demonstrates resistance to *Acacia* CT ($\leq$ 4%) *in vitro*, whereas *S. bovis* is inhibited at >0.5% (O'Donovan and Brooker, 2001). Whether *S. gallolyticus* or other rumen microbes have the ability to degrade CT in the rumen is unclear. More research is needed to better understand how CT affects rumen microbial populations and conversely how CT are affected by rumen populations.

Another key protective mechanism for the ability of animals to tolerate anti-nutritional effects of CT is the production of tannin-binding proteins in animal saliva. Proline-rich proteins have long been identified as an important component in saliva for binding dietary CT (McArthur et al., 1995) and HT (Cappai et al., 2013). Recent studies have indicated that CT do not only interact with acidic and glycosylated proline-rich salivary proteins, but also with histatins and statherins (Soares et al., 2011; Soares et al., 2012b). The aforementioned research was conducted using saliva of human source. Little research has focused on saliva of ruminant livestock. It is unclear as to which types of proteins other than those that are proline-rich occur in ruminant livestock. Mole et al. (1990) determined the proline concentration in saliva of cow, pig, and sheep sources and reported that cow saliva had the greatest proline concentration, but all demonstrated low affinity for tannin. Alonso-Diaz et al. (2012) reported the amino acid profiles of saliva of sheep and goats and found that proline was a minor component relative to histidine, arginine, and glutamate. In addition, affinities of the salivary proteins to different sources of CT differed between sheep and goats, possibly reflecting differences in feeding strategies. It is also possible that the tannin-binding capacity of salivary proteins is a temporary physiological adaptation of ruminants to conserve nitrogen and its production increases only when animals consume diets rich in CT (Vargas-Magana et al., 2013). To further complicate this matter, other dietary constituents, such as carbohydrates, may inhibit the ability of CT to complex with salivary proteins (Soares et al., 2012a). We are starting to better understand the complexity of salivary tannin-binding protein physiology, but targeted studies are still needed to determine the families of tannin-binding salivary proteins present in livestock, namely sheep, goats, and cattle, and to

unveil the associated CT binding affinities and inhibitions by other dietary constituents.

Interactions between condensed tannins and protein in the diet

A vast number of studies has documented CT-protein complexation and precipitation (Hagerman, 2012). However, due to the complex nature of the entities involved in these studies, much is yet to be interpreted regarding the control and extent of contribution that both the CT and protein make in the formation of complexes and subsequent precipitation. There is a consensus among tannin researchers that both hydrogen bonding and hydrophobic interactions between the CT and protein play an important role in the complexation and precipitation process. Although the depiction of the hydrogen bonding possibilities between these participants can be conceptualized (Figure 4), the intricacies of intermolecular hydrophobic interactions are less apparent. We have learned that, among the many factors affecting tannin-protein binding (Ozdal et al., 2013), the formation of the CT-protein complex and subsequent precipitate formation depends on the structure of both the protein (Hagerman and Robbins, 1993; de Freitas and Mateus, 2002; Lorenz et al., 2014) and the CT (Poncet-Legrand et al., 2006; Lorenz et al., 2014) under examination, the isoelectric point (pI) of the protein (Hagerman and Butler, 1981; McNabb et al., 1998), the pH of the study medium (McNabb et al., 1998; Adamczyk et al., 2012), and the tannin-protein molar ratios (Hagerman et al., 1998; Charlton et al., 2002; Adamczyk et al., 2012). Experimental evidence suggests that tannins bind to proteins, forming a tannin coating of the protein through a surface adsorption mechanism and this can lead to precipitation of the tannin-protein complex (Dobreva et al., 2011). With high tannin-to-protein ratio, the protein is coated in tannin and leads to its precipitation, whereas a low tannin-to-protein ratio encourages interlinking of soluble tannin/protein complexes, promoting aggregation of the complexes (Spencer et al., 1988; Le Bourvellec and Renard, 2012) and, ultimately resulting in precipitation of the complex.

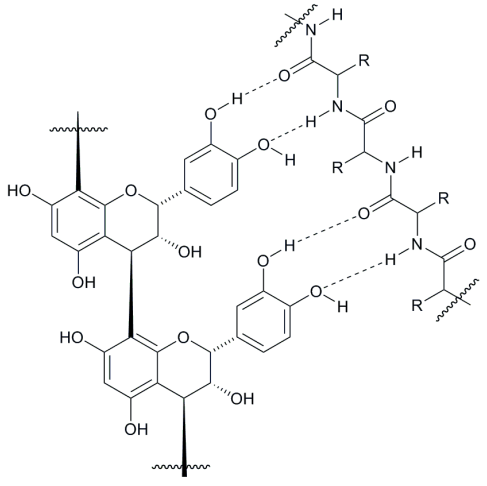


Figure 4 - Schematic representation of a hydrogen-bonding array between condensed tannin and a protein amide backbone.

The efficiency of CT-protein binding is highly variable (Hagerman and Butler, 1981; Canon et al., 2011). Once thought to be a non-specific interaction, results reported by Perez-Gregorio et al. (2014) suggest that tannin-protein binding is highly specific. However, specificity of this interaction seems to also be a function of the polarity of the tannin as well as the protein (Perez-Gregorio et al., 2014). The implications of this specificity in ruminant nutrition are complicated by the variation in concentrations and structural characteristics of the predominant proteins and CT present in the rumen (Lorenz et al., 2014) as well as rumen pH, the latter also affected by diet. Dietary constituents may be highly variable in protein types and subsequent amino acid composition, as well as in structural chemistry and types of CT present, resulting in difficulty to predict a particular animal response from CT-protein interactions. Tedeschi et al. (2014) indicated that modeling is one way to understand the complexity of CT associations in the gastrointestinal tract.

To optimize production efficiency, rumen degradable protein (RDP) must be balanced with energy availability to maximize microbial protein synthesis (Brooks et al., 2012). Post-ruminal amino acid composition can be manipulated through supplying rumen-undegradable protein (RUP) to match the amino acid requirements of the animal (Merchen and Titgemeyer, 1992; Schwab, 1995). Common dietary N sources for ruminant diets, such as *Medicago sativa* and soybean meal, are highly degradable in the rumen, resulting in high ruminal ammonia (NH_3) concentrations and low N utilization efficiency – typically around 25% (Calsamiglia et al., 2010). This inefficiency is confounded by high RDP levels that downregulate N recirculation efficiency (Agle et al., 2010) and increase blood urea N (BUN) concentrations, which are associated with reproductive and fertility problems (Elrod and Butler, 1993; Westwood et al., 2000; Roche, 2006; Tshuma et al., 2014).

Condensed tannins may offer an effective strategy to protect dietary protein from degradation through formation of stable complexes in the rumen environment (Patra and Saxena, 2011). The effects of CT from various forage

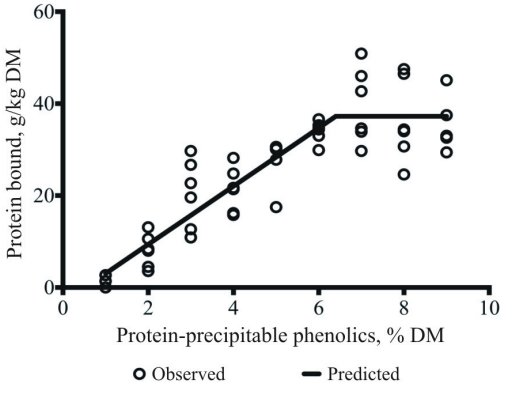
legumes on RDP are well studied, but the relationship is blurred by inconsistent experimental methodologies. In general, inclusion of dietary CT shifts the site of N metabolism and absorption by reducing both the rate and extent of ruminal protein degradation (Coblentz and Grabber, 2013) and NH_3 concentrations (McNabb et al., 1996; Min et al., 2005; Agle et al., 2010), increasing post-ruminal amino acid flow (Waghorn et al., 1994b).

Results from our research team (Naumann et al., 2014c) suggested that we can use a screening tool to predict the potential of structurally diverse mixtures of CT to bind protein based on the concentration of protein-precipitable polyphenols (Figure 5). The linear-segmented regression is represented by Equation 1:

$$PB_i = \begin{bmatrix} -3.2935 + 6.3329 \times PPP_i + \varepsilon_i \text{ if } X < 6.4 \\ 37.24 + \varepsilon_i \text{ if } X \geq 6.4 \end{bmatrix},$$

in which PB is the amount of protein bound (g/kg DM) and PPP is plant protein-precipitable phenolics (% DM). The critical percentage of dietary PPP that occurred at the intersection of the linear response and the plateau line was 6.4%. The maximum amount of protein bound by a mixture of PPP was estimated as 37.24 g protein/kg DM. Condensed tannins account for 81% of the variation that occurred in the observed values of protein bound.

It is well established that CT inclusion decreases the rate and extent of ruminal CP degradation (Waghorn, 2008; Patra and Saxena, 2011). *Lotus corniculatus* is a frequently studied CT-containing forage and is often evaluated as a replacement for *Medicago sativa*. *Lotus corniculatus* (0.97-2.77% CT) linearly decreased the immediately soluble CP fraction and rate of degradation *in situ*, thus decreasing forage RDP proportion compared with *Medicago sativa* (0% CT) (Williams et al., 2010; Coblentz and Grabber, 2013).



DM - dry matter.

Figure 5 - Linear segmented regression of protein bound (g/kg dry matter) on different concentrations of chemically diverse mixtures of protein-precipitable polyphenols (%) on a dry matter basis. Adapted from Naumann et al. (2014c).

Regressing protein degradation extent on forage CT concentration indicated that each unit of CT protected an average of 0.61 units of CP from ruminal degradation (Coblentz and Grabber, 2013).

Other effective CT-containing forages include *Lespedeza stuevei* Nutt. and *A. angustissima* var. *hirta*, which were identified as plants contributing exceptionally high protection from *in vitro* ruminal soybean meal protein degradation when mixed in a 1:1 ratio (Johnson et al., 2015). In the same study, the extent of CP degradation at 48 h was inversely related to the concentration of protein-precipitable polyphenols in six warm-season perennial legumes (Johnson et al., 2015).

Similar results are observed when CT extracts are supplemented. *Vitis vinifera* (grape) seed extract (GSE) complexed with *Lupinus angustifolius* var. *Tanjil* seed CP (at ratios of 96 and 180 mg GSE/g CP) decreased the immediately soluble fraction and CP degradation rate in Merino rams, resulting in an increased proportion of RUP with increased CT concentration (Bruno-Soares et al., 2011). However, the effective reduction of CP degradation was not proportional to the two-fold increase in CT (Bruno-Soares et al., 2011). Quebracho tannin extract (QTE) is a commercially available CT source from *Schinopsis* spp., consistently reported to decrease ruminal CP degradation at multiple dietary fiber (Dschaak et al., 2011) and CP levels (Aguerre et al., 2016). Responses appear to be dose-related as increasing inclusion from 1 to 6% of DM linearly decrease ruminal CP degradation (Ahnert et al., 2015).

Decreased protein degradation in the rumen due to CT supplementation subsequently decreases NH_3 concentrations, providing further value to the animal. Rumen available N in excess of microbial growth requirements is absorbed as NH_3, metabolized to urea in the liver, and either recycled or excreted in urine. High protein degradation rates increase urinary N excretion, which can negatively impact the environment (Powell et al., 2010), decrease N utilization efficiency, and impart a metabolic burden associated with increased urea synthesis and excretion that hinders animal performance (Van Duinkerken et al., 2005; Kohn et al., 2005) and fertility (Westwood et al., 2000; Tshuma et al., 2014).

Increasing RUP through CT inclusion consistently decreases ruminal NH_3-N concentration *in vitro* and *in vivo* across multiple species, unless feed protein levels are much greater than the requirements of the animal. A dose response is generally observed and is related to the protein precipitation ability of the CT (Johnson et al., 2015). In a continuous culture model, replacing either half or all of *Medicago sativa* hay in a dairy ration with *L. corniculatus*

hay (0.32 and 0.97% total dietary CT) decreased NH_3-N concentration and flow by at least 15 and 25%, respectively (Williams et al., 2010). Higher CT concentrations *in vivo* decreased rumen NH_3 concentration up to 24% when *L. corniculatus* silage (7.3-9.5% dietary total CT) replaced *M. sativa* silage (0% dietary total CT) in a dairy cattle ration (Hymes-Fecht et al., 2013). A few studies however, have reported no effect of CT on ruminal NH_3-N concentrations (Williams et al., 2011; Dickhoefer et al., 2016). These results may be explained by CT source, lower water intake [i.e. lower NH_3 dilution as in Dickhoefer et al. (2016)] or because energy availability was limiting for microbial growth. Christensen et al. (2015) also reported no differences in rumen NH_3-N concentration of lactating dairy cows after replacing 50 or 100% of *M. sativa* hay with *L. corniculatus* (0.51% CT). The lack of response appears to be due to high variation (1.244 mg/100 mL) given that NH_3-N concentrations were greater in the *M. sativa* diet (8.33 mg/100 mL) versus the mixed (6.05 mg/100 mL) and *L. corniculatus*-based (6.70 mg/100 mL) diets (Christensen et al., 2015). However, microbial protein production was improved in a birdsfoot trefoil diet suggesting a more efficient use of dietary N when a CT-containing forage was offered in replacement for alfalfa. As rumen NH_3 is reduced, CT shift N excretion from the urine to feces (Deaville et al., 2010; Williams et al., 2011; Hymes-Fecht et al., 2013; Ahnert et al., 2015; Orlandi et al., 2015; Aguerre et al., 2016), which may substantially improve environmental sustainability of ruminant production through reduced nitrous oxide emissions from manure (Powell et al., 2010).

The effect of CT on microbial protein synthesis and microbial growth efficiency are much less clear compared with the well-established responses on ruminal CP degradation and NH_3 concentrations. Addition of QTE at 1, 2, and 3% of DM was reported to improve microbial efficiency in sheep fed *M. sativa* hay (Al-Dobaib, 2009) and Getachew et al. (2008) reported improvements only at concentrations of 0.5 and 1%, but not 1.5%. In contrast, QTE infusion at 2, 4, or 6% of DM intake reduced duodenal microbial protein flow by 11, 21, and 39% (Dickhoefer et al., 2016) and up to 36% when QTE was supplemented at concentrations greater than 1% of DM intake (Ahnert et al., 2015).

Supplementation with CT-containing forage seems to have similar contradictory responses for different animal species. For dairy cattle, feeding *L. corniculatus* rather than *M. sativa* increased microbial protein production (Christensen et al., 2015). In contrast, multiple *in vivo* sheep trials (n = 11) using a wide range of dietary CT concentrations (0-200 g/kg DM) reported no effect on microbial protein synthesis (Min et al., 2003). Because of the important contribution of microbial protein to ruminant amino acid requirements, future evaluations of CT-containing forages or extracts should address the effects on microbial protein production and efficiency.

Initial interest in utilizing CT to improve ruminant N metabolism was based on work by Waghorn et al. (1987), who reported 50% greater post-ruminal flux of essential amino acids due to *L. corniculatus* CT followed by an average of 60% improvement in intestinal amino acid availability. The magnitude of these results has not been replicated yet. In general, CT increase post-ruminal amino acid flux due to greater RUP proportions, but effects on intestinal amino acid availability vary widely. Responses seem to depend on CT source and chemical characteristics (Kariuki and Norton, 2008), in addition to diet composition and the physiological state and production level of the animal.

Most CT-protein complexes, depending on the binding affinity, are assumed to dissociate under the acidic conditions of the abomasum, releasing both compounds into the digestion matrix (Patra and Saxena, 2011; Hagerman, 2012). Neutral pH conditions in the small intestine provide another opportunity for CT-nutrient binding, although complexes are less likely to form as the pH increases above neutral (Hagerman et al., 1992). Affinity and binding strength of CT-protein interactions affect protein digestibility throughout the digestive tract.

Kariuki and Norton (2008) directly confirmed the dissociation of proteins from CT post-ruminally in sheep. Bovine serum albumin had greater than 82% true digestibility when introduced through an abomasum cannula as a complex with CT, suggesting the majority of CT-bound protein was released and available post-ruminally. However, *L. corniculatus* CT improved post-ruminal amino acid supply in sheep, but decreased intestinal amino acid availability (Waghorn et al., 1994b). Yet, blood amino acid concentrations indicated the reduced amino acid digestibility was compensated for by the increased N flow to the intestines due to CT; thus, net absorption was not affected (Waghorn et al., 1994b).

Acacia mearnsii tannin extract supplementation of 9-17 g/kg DM to Holstein steers increased amino acid flux into the duodenum by an average of 30% above the mean value for the control treatment (Orlandi et al., 2015). Most importantly for dairy production, methionine and gluconeogenic amino acid supply was positively influenced by CT supplementation. While both apparent and true N digestibility were linearly decreased by 7% or less as CT inclusion increased, N retention was linearly improved from 17.3 to 33.2 g/day (Orlandi et al., 2015). This finding

suggests that an improvement in post-ruminal amino acid supply outweighed any impairment in protein digestibility. These data also provide some insight into CT effects on endogenous protein secretion.

Increased endogenous protein losses, primarily through increased mucus production (Sell et al., 1984) or CT complexation with digestive enzymes (Al-Mamary et al., 2001), is an often cited explanation for observed impairments in intestinal protein digestibility in both ruminants (Waghorn, 2008) and non-ruminants (Jansman, 1993). Orlandi et al. (2015) reported a similar degree of N digestibility inhibition when calculated on an apparent basis and when the effect of endogenous losses was removed by utilizing only neutral detergent insoluble N as a proxy for N of dietary origin. This suggests that *Acacia mearnsii* tannins at concentrations of 0.9-1.7% of DM did not stimulate endogenous protein secretion. Whether CT increase endogenous protein secretion apparently decreasing total tract protein digestibility, overall N retention and animal performance is the most important metric by which to evaluate CT supplementation effectiveness.

The fate of CT in the context of hypothesized CT-protein interactions remains unclear. What happens to the potentially stable CT-protein complexes as they translocate from the rumen to the abomasum and beyond? One hypothesis is that CT are depolymerized by acid hydrolysis of interflavan bonds at acidic pH encountered in the abomasum. However, if CT are not degraded in the rumen or abomasum, it is possible that CT could re-complex with any undigested proteins, peptides, or amino acids, thereby preventing absorption of these compounds in the small intestine and reducing a potential increase in amino acid absorption by the animal. This could be a possible explanation for a shift away from urinary N excretion (Grainger et al., 2009) and a decrease in urease activity and subsequent ammonia volatilization from feces (Aguerre et al., 2011) when ruminants are consuming a diet rich in CT.

In vivo experiments can help answer more practical questions relating to CT efficacy in animal production. Fully grown heifers fed a grass hay and concentrate diet with QTE added at 1-6% of the daily DM intake had reduced apparent total tract CP digestibility at dosages greater than 2%, but impaired digestibility of other nutrients when QTE was greater than 4% (Ahnert et al., 2015). Despite the negative digestibility effect, QTE inclusion improved overall N retention compared with the control, irrespective of the dosage level. This result suggests muscle growth may not be impaired as long as adequate levels of digestible energy are available. Similar responses on total tract nutrient digestibility were observed in lactating dairy Holstein cows by Aguerre et al. (2016), who reported linear digestibility decreases due to inclusion of a tannin mixture (1/3 chestnut extract, 2/3 quebracho extract) at 0.45, 0.90, and 1.80% of DM. Milk production efficiency (kg milk/DMI) was improved, but milk protein yield (kg true protein/day) decreased linearly.

Animal agriculture must efficiently utilize feed N and prevent excessive N release to the environment to achieve and maintain sustainability. Condensed tannins are a potential tool for nutritionists to achieve this goal. Although clarification is needed for CT effects on post-ruminal amino acid availability and endogenous N secretion, it is well accepted that low to moderate dietary CT concentrations slow CP degradation in the rumen resulting in increased proportions of RUP and greater post-ruminal amino acid flux. Conservatively assuming the improvement in intestinal amino acid abundance is negated by decreased apparent protein digestibility, improvements in ruminant N balance and physiology seem to be due primarily to reductions in rumen NH_3 concentrations, which consequently diminish metabolic costs associated with urinary urea excretion and inefficient N recycling. Furthermore, CT represent an opportunity to manipulate dietary RUP and target a profile of post-ruminal amino acids to more closely match requirements of animals and improve beef and dairy production efficiency.

The anthelmintic activity of condensed tannins

One of the greatest concerns of ruminant producers is that of gastrointestinal nematode parasites, especially *Haemonchus contortus*. Major costs associated with these infections include treatment with anthelmintic pharmaceuticals, losses from decreased animal production, and losses due to animal death, all exacerbated by the increasing anthelmintic resistance of parasite populations (Leathwick and Besier, 2014). Offering forages or feeds that contain CT to ruminants has potential for mitigating the gastrointestinal parasite problem. Supplementation of *Lespedeza cuneata* at 50 and 75% of the DM decreased fecal egg count, specifically of *Haemonchus contortus*, by 84.6 and 91.9% in Boer goats (Terrill et al., 2009). Minho et al. (2008) reported reductions in fecal egg count and adult *Haemonchus contortus* in the abomasum of sheep when CT extracts of *Acacia molissima* (15% CT, DM basis) was given, but no effect was observed on adult *Trichostrongylus colubriformis* in the small intestines. The anthelmintic properties of CT may not only be parasite specific, but might also be compartment specific (Tedeschi et al., 2014): some have reported greater effectiveness

of CT against gastrointestinal nematode parasites in the abomasum rather than in the small intestine.

A major challenge of using CT-containing plants as anthelmintics is that not all forages containing CT have anthelmintic properties (Naumann et al., 2014a). Condensed tannins from *Leucaena retusa*, *Lespedeza stuevei*, and *Acacia angustissima* var. *hirta* decreased larval migration of *Haemonchus contortus* by 65.4, 63.1, and 42.2%, respectively. Condensed tannins from *Desmanthus illinoensis, Lespedeza cuneata*, and *A. angustissima* were less effective against L3 larvae. Like CT-protein interactions, variation in anthelmintic activity in response to different sources of CT is probably a structure-function relationship. Quijada et al. (2015) demonstrated that CT composed predominately of prodelphinidin subunits had greater anthelmintic activity than those composed predominantly of procyanidin. This may explain why *L. cuneata*, a forage that produces CT composed predominately of prodelphinidin subunits (Naumann et al., 2015b), has demonstrated high anthelmintic efficacy *in vivo* (Shaik et al., 2006; Terrill et al., 2007; Terrill et al., 2009). Similar findings have been reported with cattle parasites. Condensed tannins composed predominantly of prodelphinidin reduced motility of L1 and adult stage *Ostertagia ostertagi* and *Cooperia oncophora* (Desrues et al., 2016).

There is enough evidence that smaller CT polymers have greater anthelmintic activity than large CT polymers (Naumann et al., 2014a, Barrau et al., 2005). However, Quijada et al. (2015) and Desrues et al. (2016) reported the opposite effect and suggested that variation in results is likely due to differences and difficulties in extraction and purification of CT, as well as differences in modes of action of different CT on different gastrointestinal nematode parasites. The mode of action of CT on gastrointestinal nematode parasites has not been entirely clarified. However, electron micrographs of nematode larvae following incubation with CT extract show alteration of the nematode cuticle and binding of CT in the cephalic region (Hoste et al., 2012).

Condensed tannins in combination with other plant specialized metabolites may impact the efficacy of these compounds as anthelmintics. Klongsiriwet et al. (2015) demonstrated synergistic effects of combining flavonoids such as quercitin and luteolin with CT. The anthelmintic activity of CT was enhanced in the presence of flavonoids using an *in vitro* larval exsheathment assay.

Using HT may also have promise as a potential anthelmintic. Engström et al. (2016) demonstrated that many HT have little if any anthelmintic efficacy. However, those composed primarily of the PGG structure demonstrated inhibition of egg hatching and larval motility. Evidence suggests that the external structure of the egg, larval midbody, and cephalic region are altered in the presence of HT (Engström et al., 2016).

The interactions between minerals and condensed tannins

In addition to the ability to bind to and precipitate proteins, tannins and other polyphenols efficiently bind to Fe and, to a lesser extent, to Cu, Mn, Al, Zn, and Co. There is great variability in the extent to which polyphenol-metal chelation affects mineral bioavailability. Yet, evaluations of CT supplementation in both ruminants and monogastrics rarely address the potential effects on mineral nutrition. While CT effects on mineral availability *in vivo* remain vague, the chemistry behind the phenomenon is better understood. The presence of at least two adjacent hydroxyl groups on a phenyl ring (ortho or *o*-dihydroxyphenyl group) are the minimum requirements for mineral chelation (Andjelkovic et al., 2006). This structural chemistry contributes to plant pigmentation as well as cation-nutrient cycling throughout the environment of the plants (Quideau et al., 2011). To generalize, the more *o*-dihydroxyphenyl functional groups in a CT molecule, the greater potential for metal chelation (McDonald et al., 1996). The interaction is pH dependent and greatly influences CT anti-oxidative capacity (Kumamoto et al., 2001). Iron and Al cations bound to CT at pH levels of 3.20 or less, whereas Mg, Ca, Zn, Cu, Mn, and Co cations bound to CT at a pH greater than 3.70 (Faithfull, 1984). However, it is generally accepted that these complexes are stable over a wide pH range and throughout the entire gastrointestinal tract (McDonald et al., 1996; Kumamoto et al., 2001; Scalbert et al., 2002).

Iron is the most frequently studied mineral in relation to tannins due to strong Fe-binding potential. Early studies investigating iron absorption inhibition by phenolic compounds indicated a close relationship between the amount of tannin added to a meal and the degree of inhibition (Brune et al., 1989). Since then, functional groups important for iron and other mineral binding have been identified. Tannins with catechol and galloyl groups are effective metal chelators (Andjelkovic et al., 2006; Perron and Brumaghim, 2009). Each CT molecule may bind two or more metal ions and each metal ion may form chelates with *o*-dihydroxyphenyl groups from two different CT molecules (McDonald et al., 1996). A 3',4'-dihydroxygroup on a flavonoid B-ring (Figure 6) is required for Fe binding (Khokhar and Owusu-Apenten, 2003) and increased free hydroxyl groups are associated with increased Fe-binding ability (Andjelkovic et al., 2006; Mladěnka et al., 2011).

Condensed tannin-Fe complexes can effectively inhibit Fe absorption (Lavin et al., 2010; Wren et al., 2013) at levels as low as 5 g tannic acid/kg (Afsana et al., 2004). In cell culture, a 1:1 ratio of tannic acid to Fe inhibited 92% of Fe absorption (Glahn and Wortley, 2002). When applied in a more complicated feed matrix as part of a meal, this effect was lessened, but still evident (Yun et al., 2004), suggesting that degree of mineral absorption inhibition is influenced by the presence of other nutrients in the feed matrix during digestion. Ascorbic acid and ethylenediamine tetraacetic acid enhance Fe bioavailability by reversibly or irreversibly forming a more soluble complex with Fe as opposed to the complexes formed by CT. Diets containing ingredients with high ascorbic acid concentrations enhance Fe absorption and efficiently inhibit CT-Fe chelation even at low concentrations (Tamilmani and Pandey, 2016).

Condensed tannins bind Cu through similar chemical mechanisms. As with other cations, CT chemical structure, in particular the type of hydroxyl substitution, may explain differences in their ability to chelate Cu. Pyrogallol precipitates Cu more efficiently than catechol hydroxylation patterns and precipitation is reduced as pH decreases (McDonald et al., 1996). Contrary to previous reports, tea tannins have been shown to increase Cu bioavailability in rats (Scalbert et al., 2002).

Zinc has a much lower affinity for CT than Fe and Cu (McDonald et al., 1996), particularly at acidic and neutral pH conditions (Santos-Buelga and Scalbert, 2000). Afsana et al. (2004) demonstrated no effect of tannin supplementation on Zn absorption *in vivo*. Reports of CT complexes with other cations are scarce and usually related to tea consumption. Tea tannins have been reported to inhibit Al absorption in humans and rats (Fairweather-Tait et al., 1991), but was found to have no effect in other studies (Greger and Lyle, 1988).

In addition to direct binding, cations may also influence CT interactions with dietary proteins. Calcium has been found to enhance the protein binding capacity of tea tannin, epigallocatechin gallate, with β-lactoglobin in milk, allowing the formation of larger CT-protein complexes (Carnovale et al., 2015). The effects of CT on vitamin absorption and metabolism are not well understood. However, it may be an important consideration because tannic acid has been shown to negatively impact vitamin A status in rats and may interact with thiamin and reduce vitamin B absorption (Jansman, 1993).

Interaction of CT with metal ions not only influences mineral bioavailability, but is one of the mechanisms by which CT act as potent antioxidants in the intestinal lumen (Brenes et al., 2008; Perron and Brumaghim, 2009). In face of increasing restrictions on the use of antibiotics in animal production, interest in CT application to improve gut health (Redondo et al., 2014) is rapidly increasing. Yet, information regarding interactions between CT, vitamins, and minerals *in vivo* is lacking. To an extent, it is reasonable to assume mineral status is adequate so long as animal performance is not impaired. However, CT research for application to animal production should consider mineral balance due to the strong iron chelation potential of CT and because of any number of nutrient interactions that may occur.

Current reports on the influence of CT on ruminant mineral nutrition are quite inconsistent. Studies by Waghorn et al. (1994a), evaluating the effect of CT in fresh cut *Lotus pedunculatus* (5.5% CT) given to sheep reported that CT reduced S absorption and increased net absorption of P and Zn, while effects on other minerals (Fe, Cu, Ca, P, and Mg) were small or not affected. Pine bark CT (0.06-1.11% of DM) linearly decreased digestibilities of K, S, and Cu, whereas P, Mg, Mn, Zn, and Fe digestion was increased in meat goats (Min et al., 2015). It is possible that some minerals bound by CT in the rumen become available in the small intestine. Pagan-Riestra et al. (2010) demonstrated that a shift in P disappearance from the rumen to the small intestine was related to the presence of biologically active CT from *A. angustissima*.

Condensed tannin effects on monogastric mineral balance are more frequently reported and mineral availability generally decreases with increasing CT concentration. Broiler chicks consuming diets with increasing, but low levels, of GSE (0.025, 0.25, 2.5, and 5 g/kg) had linearly decreased plasma concentrations of Cu, Fe, and Zn (Chamorro et al., 2013). High-tannin sorghum (1.36% CT)

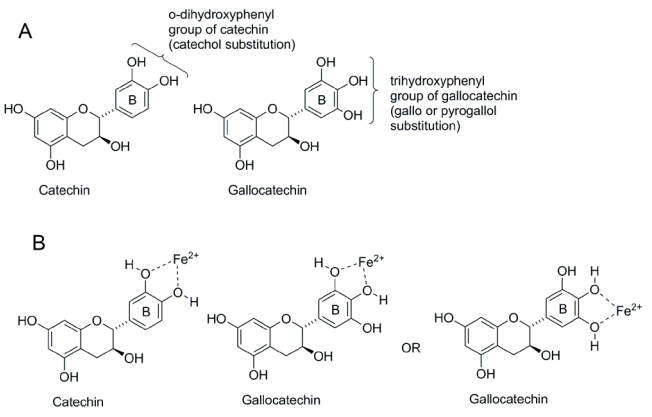


Figure 6 - Phenolic hydroxyl substitution patterns for catechin and gallocatechin (A) and phenolic hydroxyl-ferrous iron binding modes for catechin and gallocatechin (B).

reduced apparent absorption of Ca, P, Mg, Na, K, Fe, and Co in broilers (Hassan et al., 2003). Similarly, mineral digestibility linearly decreased as sorghum tannin inclusion increased to 3% of diet DM (Mahmood et al., 2014). Few reports are available regarding CT supplementation in swine diets in general and those measuring effects on mineral digestibility are even rarer. Tannic acid, a hydrolisable tannin, has been shown to reduce Fe availability in weanling pig diets (Lee et al., 2010). Condensed tannins have been used effectively to inhibit iron absorption and mitigate iron overload disorder in multiple exotic species (Wood et al., 2003; Lavin et al., 2010; Lavin, 2012).

A final consideration is the contribution of CT-containing ingredients to diets. The ability of CT to chelate metals could promote the accumulation of metals either from the growing environment of the plant or through diet processing. In this scenario, the CT could become contributors of metal ions to the animal, rather than reducing metal ion availability as a metal-ion chelator. This would be problematic for prevention of iron-overload disorder or other mineral toxicities. As research in CT and ruminant interactions progresses, it will be important to measure and report the implications of CT on mineral balance in addition to N metabolism and overall performance to ensure the safety of long-term CT supplementation.

CT-enteric fermentation interactions

Methane is a potent greenhouse gas that is produced normally during microbial fermentation in the rumen and released to the environment during eructation. Methane gas produced by livestock represents the second greatest source of CH_4 to the atmosphere, estimated at 22% of total anthropogenic CH_4 emissions in the US; beef and dairy cattle are responsible for 96% of these emissions (USEPA, 2016). However, when expressed as CO_2 equivalents, the livestock sector is responsible for 4.2% of total greenhouse gas emissions; beef and dairy cattle are the primary drivers of CH_4 production from the livestock sector, representing only 3.6% of the total contribution (USEPA, 2016).

Methanogenesis is a complex process by which methanogens in the rumen digest cellulose into forms usable by the animal. Buddle et al. (2011) developed a simplified diagrammatic representation of this process in which rumen bacteria, protozoa, and fungi act upon feedstuffs entering the rumen. Metabolic byproducts from rumen microbes include H_2, CO_2, and volatile fatty acids (VFA), among others. Enteric CH_4 is produced during the disposal of metabolic H_2. Reducing equivalents that are not consumed during the formation of volatile fatty acids may be used to produce CH_4, representing a loss of metabolizable energy to the animal.

One of the many important symbiotic associations formed in the rumen is that of the relationship between methanogenic archaea and ciliated protozoa (Ng et al., 2016). It has been proposed that this association occurs to facilitate interspecies transfer of metabolic H_2 from protozoa to methanogens. Carbon dioxide is reduced by methanogens to produce energy, creating CH_4 as a metabolic byproduct in the following way: $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$. It is also postulated that H_2 in combination with CO_2 could be utilized by homoacetogens to produce acetate, a primary energy source for the ruminant animal. Thus, if less H_2 is converted to CH_4, then more H_2 is available for VFA production, resulting in an increase in metabolizable energy for the animal.

Feeding CT-containing forages or feedstuffs to ruminants may be an effective natural practice for mitigating CH_4 emissions by ruminant livestock and increasing metabolizable energy intake. A diverse group of legume species with varying types of CT inhibited CH_4 production *in vitro* (Naumann et al., 2013b). However, when these CT-containing legumes were fermented as the sole source of forage, they also inhibited total gas production and VFA production, indicating an inhibition of digestibility. This suggests that inhibiting CH_4 does not result in a shift in H_2 from methanogens and methanogenesis to homoacetogens and VFA production and demonstrates a downside of using CT for CH_4 mitigation. The objective should be to decrease CH_4 emissions from ruminant livestock without compromising production, which requires selectively reducing CH_4 production without subsequent reductions in total gas or fermentation. It is possible to accomplish this objective with CT. In *in vitro* rumen fermentation studies, replacement of 45% of the forage portion of a corn-alfalfa diet for either *L. cuneata* or *D. paniculatum*, achieving dietary CT concentrations of 2.6 and 9%, respectively, decreased CH_4 production without decreasing total gas production (Naumann et al., 2015b).

The mechanism by which CT impact methanogenesis and reduces CH_4 production by ruminants is not well understood. There are multiple hypotheses for how CT inhibit methanogenesis, none of which have been definitively proven. One hypothesis is that CT act directly upon methanogens in the rumen. Ng et al. (2016) reported the existence of a protein-based adhesin probably located at the tips of fimbriae that function in facilitation of the methanogen-protozoa symbiosis. Parts of the cell envelope, including the cell membrane, wall, and glycocalyx, also contain protein. It is possible that CT bind

to this proteinaceous adhesin or parts of the cell envelope, interfering with establishment of the methanogen-protozoa complex and decreasing interspecies H_2 transfer. Inhibition of this symbiotic relationship may also negatively impact the ciliated protozoa population. Bhatta et al. (2015) determined that rumen ciliated protozoa populations decreased when the feedstuff contained CT from *Ficus bengalensis* and *Azardirachta indica* at concentrations of 26 and 13.8%, respectively.

Another hypothesis is that indirect inhibition occurs by decreasing the availability of nutrients to rumen microorganisms, subsequently reducing substrate digestibility and indirectly inhibiting rumen microbial populations. Because CT bind to minerals (Lavin, 2012) and organic molecules such as proteins (Saminathan et al., 2014), carbohydrates (Soares et al., 2012a), and lipids (Delehanty et al., 2007), it is possible that not only do these complexes become unavailable as substrate for use by rumen microbes, but that CT bind to microbial enzymes modulating their activity (Gonçalves et al., 2011). Naumann et al. (2013c) demonstrated a weak relationship between protein bound by CT and a decrease in CH_4.

A third hypothesis for how CT inhibit CH_4 is that CT act as a hydrogen sink (Naumann et al., 2013a). Becker et al. (2014) reported an abatement of methane production in an *in vitro* rumen fluid environment that occurs linearly with the addition of flavan-3-ol catechin. In this experiment, as many as six hydrogen atoms per catechin molecule were captured by catechin-degradation products and CH_4 production was reduced at a rate of 1.2 mol CH_4 per mol catechin. From this study, the authors reported characterization of metabolism products arising from catechin acting as a hydrogen atom acceptor (hydrogen sink). These findings parallel compounds isolated from biotransformations of flavan-3-ols by human microflora (Feng, 2006; Monagas et al., 2010). Briefly, catechin is converted to transient intermediate 2**,** which accepts two hydrogen atoms to provide reduced compound 3 (Figure 7). Compound 3 accepts an additional two hydrogen atoms as hydrogenolysis of the C-4 phenolic bond occurs, reducing it to a C-H bond and giving rise to compound 4. Compound 5 can be derived from transient intermediate 2 through scission of the A-ring, leading to excision of a molecule of acetoacetic acid. Compound 5 accepts two more hydrogen atom equivalents undergoing dehydroxylation of the C-4 phenolic group of the B-ring to provide compound 6. Compound 3 can undergo A-ring scission, from which the intermediate hydroxyacid undergoes lactonization to deliver compound 7. Alternatively, reduction of the ketone functionality in compound 5 leads to the same intermediate hydroxyacid as derived from 3 and provides lactone 7. Hydrogenolysis, requiring two hydrogen atom equivalents, of the C-O bond of the lactone in 7 provides phenolic acid 8. Hydrogenolysis of the C-4 phenolic bond in compound 8, again requiring two hydrogen atom equivalents, provides phenolic acid 9. Although intermediates were not detected in this study to support its subsequent transformation to compound 9, compound 4 presumably could undergo biotransformations similar to those proposed for the conversion of compound 3 to compound 9 (A-ring scission/ lactonization, lactone hydrogenolysis) to deliver compound 9. Lastly, compound 6 could presumably be converted to compound 9 through a reduction/lactonization followed by lactone hydrogenolysis sequence. All structures (Figure 7), with the exception of compound 2, were either confirmed through comparison with commercially available materials or characterized by mass spectrometry and nuclear magnetic resonance spectroscopy.

Predicting ruminal CH_4 production is important for ruminant nutrition, especially for the estimation of metabolizable energy intake to assess the total gross energy available for metabolism by an animal. Whether driven by interests in increasing efficiency of animal production via increasing metabolizable energy intake or in making efforts to negatively impact climate change, the need for mitigation is beneficial. Predicting enteric CH_4 inhibition by dietary constituents is important for aiding in meeting mitigation or reduction targets without compromising

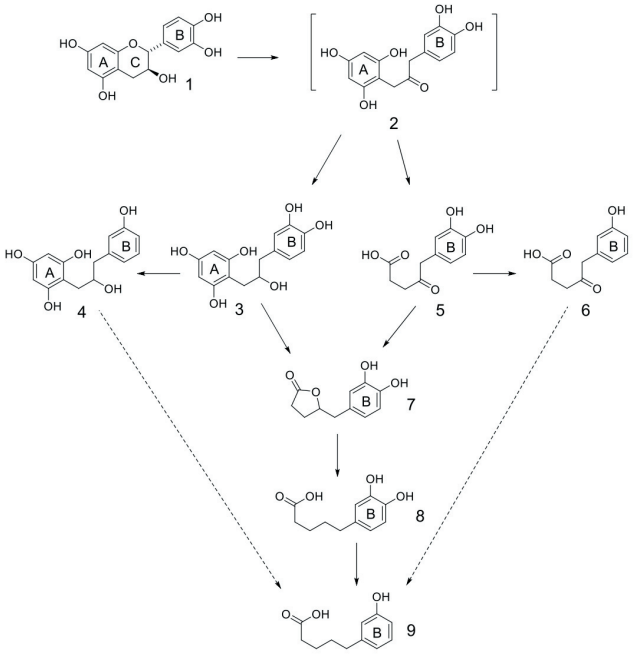


Figure 7 - Catechin as a hydrogen sink during *in vitro* rumen fermentation. Adapted from Becker et al. (2014).

animal production; the objective for the livestock sector is reduction, not complete suppression of enteric CH_4.

Equations have been developed to predict ruminal CH_4 production based on dietary constituents or various animal-related variables. For example, Dittmann et al. (2014) developed predictive equations describing the relationship between body mass and daily CH_4 emission by ruminants and camelids using linear regression. Body mass explained 93% of the variation observed in CH_4 emissions by ruminants and 91% for that of camelids. Both linear regressions demonstrated positive correlation such that, as body mass increased, CH_4 emissions increased. While the correlations are strong, one of the challenges associated with these equations is that there is no identification of the participatory factors driving the CH_4 variable. One of the probable drivers of the relationship between body mass and CH_4 is dry matter intake (DMI). As animals increase in size, the amount of DMI increases, which could increase fermentable organic matter and subsequently enteric CH_4 production. Another challenge would be applying body mass as a variable to mitigation of CH_4. To do so would result in selecting smaller animals in an effort to decrease CH_4 emissions.

Ramin and Huhtanen (2013) developed linear and quadratic equations for predicting daily enteric CH_4 production based on DMI. Intake was positively correlated with CH_4 production. For the linear regression, DMI explained 85% of the variation in CH_4 emission. However, determination of the participatory factors most closely related to enteric CH_4 production is needed for application of DMI to mitigation efforts. For example, Grainger and Beauchemin (2011) developed equations for predicting enteric CH_4 yield from cattle and sheep on the basis of dietary fat. Enteric CH_4 production was negatively correlated with total dietary fat. The predictive equation for sheep had a greater negative slope than that for cattle, suggesting that dietary fat may be more effective at mitigating CH_4 in sheep than in cattle.

Mangino et al. (2003) developed the cattle enteric fermentation model to predict CH_4 emissions based on gross energy intake. Interestingly, the predictive equation included a methane conversion factor, which was based on the fraction of feed gross energy converted to CH_4. The conversion factor was specific to characteristics of the diet and type of animal in different regions of the US. For example, the methane conversion rate for dairy cows in California was 4.8 compared with 5.8 for dairy cows in the West. Methane conversion rates only differed for dairy cows and dairy heifers, suggesting that different classes and kinds of beef cattle convert gross energy to methane at the same rate regardless of regional differences and animal type.

While much research has been conducted to evaluate the potential of forages that produce CT to decrease enteric CH_4 production, little if any research has focused on predicting inhibition of CH_4 emissions based on CT content of feedstuffs. Naumann et al. (2013b) evaluated the relationships among CT, VFA, and enteric CH_4 production. A decrease in the acetate:propionate ratio resulting from an increased use of hydrogen for propionate formation may be related to the inhibition of CH_4 production by CT. However, no relationship was observed between CT concentration and the acetate:propionate ratio ($R^2 = 0.01$) or the acetate:propionate ratio and CH_4 production ($R^2 = 0.14$). However, CT and total VFA were negatively correlated ($R^2 = 0.52$), whereas total VFA and CH_4 were positively correlated ($R^2 = 0.68$). The simplest measure of these proposed relationships is CT concentration.

Thus, can CT concentration be a satisfactory predictor of enteric CH_4 abatement in ruminants? Initial observations suggested that the amount of CH_4 produced by fermentation of each legume species varies, even among those species with the same concentration of CT (Naumann et al., 2013b). However, linear regression indicated that CT and CH_4 production were negatively correlated ($R^2 = 0.44$) (Naumann et al., 2013b,c). Only a limited number of data sets collected under similar conditions were used to test this relationship. The following nonlinear exponential decay regression equation for predicting CH_4 inhibition by CT was developed using an increased sample size: $CH_4 = 113.6 \times \text{Exp}(-0.1751 \times CT) - 2.18$; ($R^2 = 0.53$) (Naumann et al., 2015a). This equation can be used to predict the concentration of potential CH_4 produced from fermentation of substrates containing known concentrations of CT (e.g. 4% CT = 50% reduction in CH_4) and further used as a tool in precision diet formulation.

It is incumbent upon the research community to develop mathematical models that will aid in the mitigation of CH_4 production and emission by ruminants in an effort to increase the efficiency of animal production, while minimizing the impact of production on the environment. Using predictive models to estimate the impact of feed and forage constituents such as CT on ruminal CH_4 production and emission is one approach. Condensed tannins may impact the process of methanogenesis in ruminants, resulting in inhibition of CH_4 emissions. The question is: how can we predict ruminal CH_4 inhibition by CT and develop future improvements to existing methodologies?

Limitations and future directions

Probably, the greatest limitation in advancing knowledge of CT-animal interactions is related to the structural diversity of CT and its chemical determination. As described above, CT are assembled through the joining of a subset of flavan-3-ol subunits through a few different covalent bonding patterns. So, how many unique chemical entities (isomers) can be derived from a small collection of flavan-3-ol subunits and a defined set of interflavan linkages? Calculation of the number of potential isomers in a CT oligomer/polymer (Table 1) was performed using the equation ($A^m \times B^n$) = number of possible isomers, in which A = number of different flavan-3-ol subunit types in the compound, m = the actual number of flavan-3-ol subunits in the compound, B = the number of different types of interflavan linkages present in the compound, and n = the actual number of interflavan linkages present in the compound. Similar formulas have been reported to determine the number of CT structural isomers (Cheynier, 2005; Nam et al., 2017). The number of different unique compounds that could arise from CT containing two different flavan-3-ol subunits and only two types of interflavan linkages is listed in column A (Table 1). The CT from *Vaccinium* (cranberry) and *Sorghum* would fall into the category of compounds listed in this column for chemical structural isomers. *Vaccinium* and *Sorghum* CT are almost exclusively composed of procyanidin (catechin and epicatechin) subunits. Both *Sorghum* and *Vaccinium* CT possess 4-8 B-type interflavan linkages. *Sorghum* may also contain 4-6 B-type linkages and cranberries are known to contain A-type interflavan linkages. The CT from common forages such as *L. corniculatus, L. pedunculatus*, and *O. viciifolia* contain a varying mixture of PC and PD subunits, increasing the complexity of the CT (Table 1, Columns E and F). To add to the complexity, CT structures isolated from some sources, such as *Vitis* or *Diospyros* (Tian et al. 2012), are found to contain a derivatized C3 hydroxyl group as the gallate ester. This may occur multiple times and at any point along the oligomer/polymer chain. The numbers listed in Table 1 also do not take into account that occasionally enantiomers (mirror images) of flavan-3-ol subunits are isolated and characterized. Both of these occurrences would greatly increase the number of possible structural isomers and add to the challenge of identifying and characterizing CT from plant materials.

Another limitation is that much of the published research focusing on CT-animal interactions has been conducted using *in vitro* techniques. *In vitro* techniques are a satisfactory screening tool for CT bioactivity and are often simpler, more controlled, and less costly as compared with *in vivo* methods. However, there are limitations associated with results from *in vitro* methods and their correlation to on-farm realities. They do not account for microbial turnover in rumen fluid, passage rate, and removal of end products. The lack of CT-animal interaction studies using *in vivo* methods is a limitation to progress in this regard. For example, further evaluation of the nonlinear exponential decay regression equation for predicting CH_4 emissions from ruminants using live animals is needed. Today, we have a method for predicting the inhibition of CH_4 by CT. We need a robust model based on *in vivo* studies.

Short-term studies are another limitation to advancing knowledge of CT-animal interactions. Odenyo et al. (1997) demonstrated how rumen microbes adapt to plant specialized metabolites. It is possible that responses of anthelmintic activity or decreased CH_4 emissions are not sustainable due to nematode and microbial population adaptation, respectively. One of the few long-term *in vivo* studies conducted consisted of feeding *Castanea sativa* and *Quercus valonea* to sheep for 190 and 85 days, respectively (Wischer et al., 2014). Enteric CH_4 abatement was not

Table 1 - Number of possible condensed tannin isomers dependent on number of unique flavan-3-ol subunits and interflavan bond types present

	A	B	C	D	E	F
Number of flavan-3-ol units	Two units Two bond types	Two units Three bond types	Three units Two bond types	Three units Three bond types	Four units Two bond types	Four units Three bond types
2 (dimer)	8	12	18	27	32	48
3 (trimer)	32	72	108	243	256	576
4 (tetramer)	128	432	648	2187	2048	6,912
5 (pentamer)	512	2592	3888	19,683	16,384	82,944
6 (hexamer)	2048	15,552	23,328	177,147	131,072	995,328
7 (heptamer)	8192	93,312	139,968	1,594,323	1,048,576	11,943,936
8 (octamer)	32,768	559,872	839,808	14,348,907	8,388,608	143,327,232
9 (nonamer)	131,072	3,359,232	5,038,848	129,140,163	67,108,864	1,719,926,784
10 (decamer)	524,288	20,155,392	30,233,088	1,162,261,467	536,870,912	20,639,121,408

sustained for the duration of the experiment. However, digestibility decreased for some dietary constituents. The plants used in this study were rich in HT rather than CT, which may explain the anti-nutritional effects and lack of sustained decrease in CH_4. Long-term *in vivo* studies using CT, rather than HT, of known chemical and structural characteristics are needed to better understand CT-animal interactions and associated animal responses. However, researchers are limited by the lack of availability of large quantities of substrates that contain adequate concentrations of bioactive CT necessary to conduct long-term *in vivo* studies. One solution to this problem is the use of CT-rich industry byproducts such as grape pomace, a wine-industry byproduct. However, the inconsistency of such products presents additional challenges and requires CT analysis prior to each use.

Future research should also focus on CT biological activities when other plant-specialized metabolites are present. For example, predictive equations should be developed when other potential reducers of ruminal CH_4 are used in conjunction with CT. There are potential interactions of CT with other plant constituents that can potentially reduce CH_4 production (Tedeschi et al., 2011). In addition to CT, plants may produce organic acids, terpenes, and their derivatives, alkaloids, and cyanogenic glycosides among others. These metabolites combined with factors of dietary fat, fiber, and protein play a participatory role in overall ruminant nutrition, anti-nutrition, and possibly the efficacy of CT. Determining how these different plant-specialized metabolites and other dietary constituents interact in the rumen is important. Are these effects additive, synergistic, or antagonistic with respect to animal nutrition (or anti-nutrition), nutrient utilization, mineral binding, the process of methanogenesis, and anthelmintic activity?

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