AN INVESTIGATION OF SAINFOIN CONDENSED TANNIN DYNAMICS IN MANURED PERENNIAL FORAGE PRODUCTION

BY

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ABSTRACT

Condensed tannins (CT) play an integral role in terrestrial nutrient cycling. Despite being the fourth most abundant terrestrial biochemical product, the regulatory processes of tannin production in plants and their subsequent ecological influences are not completely understood. The defining characteristic of CT's is their affinity for proteins, though they willingly interact with minerals, carbohydrates, and other polyphenolic compounds. Previous tannin investigations in agriculture have centred on bioactivity related to ruminant digestive physiology and pathology. These studies have revealed that CT's have the potential to increase liveweight gain, wool production, and sheep ovulation rates, prevent pasture bloat, reduce enteric and stockpiled manure greenhouse gas (GHG) emissions, and control gastrointestinal parasites. The present study has explored how tannin-containing resident plant material and applied manure derived from tannin-containing beef cattle diets influences tame forage biomass yield, proximate analysis, and tannin production, as well as residual fall soil nutrient status, in southern Manitoba. To test these hypotheses, we conducted an experiment over two growing seasons (2007-2008) where in the fall of 2007, both tannin-derived (sainfoin, *Onobrychis viciifolia*) and non-tannin (alfalfa, *Medicago sativa*) composted beef manures were applied at a rate of 44.8 t/ha in a randomized split plot fashion, including a manure-free control, on a four repetition randomized complete block design which included both tannin-containing and non-tannin containing forage monocultures and mixtures. Plant samples were harvested in two cuts, and soil measurements were taken in the fall of both years. It was found that across all sainfoin treatments, manure origin did not have a significant effect on plant CT concentration, which was extremely variable within

treatments and cuts. Over four harvest dates, sainfoin CT concentrations ranged from 14.1 g/kg to 91.9 g/kg in monoculture plots, and 16.6 g/kg to 123 g/kg when grown in a mixture with meadow brome. In most cases, the presence of either manure type did not significantly affect soil nutrient status or forage yield, though results were similarly variable. Using a stepwise regression which included all soil and plant measurements across all cuts and treatments, it was found that NDF ($R^2 = 0.548$) and plant phosphorus ($R^2 = 0.126$) were the only significant model contributors to tannin concentration in sainfoin at $P < 0.15$. These findings suggest that nutrient effects of beef cattle manure are not realized in either plant or soil in the year following application, and consequently, that tannin agronomy requires longer-term analysis.

FOREWARD

"For nitrates are not the land, nor phosphates; and the length of fiber in the cotton is not the land. Carbon is not a man, nor salt nor water nor calcium. He is all of these, but he is much more, much more; and the land is so much more than its analysis. The man who is more than his chemistry, walking on the earth…that man who is more than his elements knows the land that is more than its analysis."

- John Steinbeck, *The Grapes of Wrath*

1.0 INTRODUCTION

Tame forage production plays a large role in shaping the agricultural landscape of the Canadian prairies. In 2006, almost 5.7 million hectares of land across Canada were devoted to tame or seeded pastures, of which over 90% was located in the four western provinces (Statistics Canada 2006). This accounted for 8.4% of total improved and unimproved arable land nationwide. Similarly, over 7.9 million hectares, or 11.8% of total agricultural land, was sown to tame hay, which represented a 55.8% increase in area over the preceding 25 years. Of these hay lands, 63.7% were sown to either alfalfa or alfalfa-grass mixtures (Statistics Canada 2006b). The sum of tame grazing and hayed lands accounts for over 20% of total agricultural areas in Canada, thus any potential improvement to either the productivity, feed efficiency, or the ancillary environmental benefits of tame forages would have a significant, widespread impact.

The increased prominence of grazed tame perennial forages as a preferred livestock feeding strategy is supported on several production and environmental fronts. Including perennial forages in Canadian prairie farming systems is an agronomically beneficial practice (Entz et al. 2002) that improves a multitude of parameters in farming systems sustainability indices (Dalsgaard et al. 1995; Pacini et al. 2003; Rao and Rogers 2006). High-producing perennial forages also provide what Robertson and Swinton (2005) describe as “environmental services”, such as more accountable nutrient cycling (Boody et al. 2005), and carbon sequestration (Baron et al. 2002) leading to atmospheric greenhouse gas reduction (Follett et al. 2001). The agronomic and ecosystem health observed in forage-based livestock production is consequently reflected in the improved

nutrient profiles of their end products (Cordain 1999; Cordain et al. 2002) leading to improved health of consumers (Ramsden et al. 2009). By improving the productivity of forage crops, the efficiency of forage feeding, then quantifying and valuing the ancillary environmental services provided by perennial forages, researchers are improving the sustainability and health of livestock production from "gate to plate". A novel strategy to achieve these aims is the integration of tannin-producing leguminous forages such as sainfoin into ruminant livestock diets.

Tannins have been studied as bioactive plant products for centuries, however, only recently have research paradigms shifted from tannins as derivative or extracted plant products to acknowledging tannins as an integral component of forest, arctic, and agricultural ecosystems. Although a considerable body of tannin literature (see Aerts et al. 1999; McMahon et al. 2000; Schofield et al. 2001; Kraus et al. 2003; Mueller-Harvey 2006; Waghorn 2008) has been assembled in a relatively short timeframe, the complex biological relationships of tannins have posed unique challenges to understanding and evaluating their potential uses and interactive roles in highly-productive agricultural ecosystems.

Low to moderate levels of condensed tannins in ruminant diets have shown to increase protein utilization (Waghorn 2008) and liveweight gain (Karnezos et al. 1994), prevent pasture bloat (McMahon et al. 1999; Wang et al. 2006), reduce both gastrointestinal parasite loads in sheep (Niezen et al. 1996; Hoskin et al. 2000) and pathogen survival in bovine feces (Berard et al. 2009), and potentially reduce enteric greenhouse gas emissions (Bouchard, unpublished; Martin et al. 2010). Similar research in forest ecosystems has shown that tannins can exert effects upon nitrogen cycling

(Talbot and Finzi 2008; Kraus et al. 2004b) and by association, organic carbon degradation rates (Schimel et al. 1996; Northup et al. 1998). Quantifying the relative importance of tannins in these ecosystem and global processes is dependent upon improved methods to determine concentrations and inputs of tannins in terrestrial biomass (Kraus et al. 2003).

Integrated, multi-disciplinary research programs addressing this diverse suite of issues will facilitate a comprehensive understanding the roles of tannins in commercially-relevant agricultural production. This framework will, in turn, provide guidance for improved methodology in tannin quantification, characterization, and manipulation for productive and sustainable ends. Such synergy between *in vivo* field operations and *in vitro* laboratory support for field observations forms the backbone that supports systems-level agricultural research.

The study of tannins through *in vivo* agronomic experiments in the Canadian prairies is a novel field. Given that plant tannin production is significantly affected by changes in soil nutrient status (Barry and Forss 1983), climate (Barry and Duncan 1984), and inter-specific competition (Wen et al. 2003), a representative study of tannin production and cycling within a simulated tame hay pasture is valuable. Understanding the potential biological interactions within this relatively undeveloped approach to tannins in systems research demands input from a diverse spectrum of scientific disciplines which are not typically cross-referenced. It is hoped that this multi-disciplinary approach to interpretation in the present study provides greater context for further study and integration of tannins into Canadian prairie agriculture.

2.0 LITERATURE REVIEW

2.1 Introduction

Haslam (1994) best described the chemical nature of plant polyphenols as:

"A veritable minefield in itself for the chemist, let alone the unsuspecting ecologist".

Despite a long-lasting recognition that tannins play a role in many biological processes, and that this role may be exploited for utilitarian purposes, their complexity and extensive structural and chemical variability in nearly all terrestrial ecosystems has stymied the deductive methods of researchers for decades.

Tannins in agriculture have recently been a topic of multi-disciplinary research interest since the mid-1960's. Though often novel in application, this interest in tannin function is not unique; these mysterious polyphenolic compounds have captured the imagination of scientific researchers for over two hundred years. In the second volume of the late Professor Henry Trimble's seminal reference work *The Tannins*, published in 1892, he established a catalogue of tannin-related reference materials exceeding 1000 entries. Though many of these studies focused on the use of tannins as a protein-binding agent in leather manufacture, the concept of harnessing the inherent bioactivity of tannins for anthropogenic purposes predates the discovery of electromagnetism and the invention of wood-pulp paper manufacture. Professor Trimble captured the quandary of this interest in proclaiming that 'The tannins occupy a part of the borderland in science between botany and chemistry' (Trimble 1892).

When travelling through scientific disciplines, the term "tannin" evokes a range of definitions which vary in specificity and perhaps more importantly, in biological function

within each area of study. The level of detail required by a food scientist, interested in the specific stereochemistry and monomeric composition of proanthocyanidins and their subsequent effects on the body of red wine, would be foreign to the forest ecologist studying the respiratory effects of total phenolics in an organic litter layer. Comparatively, since the interrelationships between a forage crop breeder and a ruminant physiologist are based on nutritive value and palatability of the feedstuff, discussions on tannins often involve common functional interests and by this, common language.

This thesis is based largely upon the production and subsequent effects of condensed tannins in sainfoin (*Onobrychis viciifolia*) in resident plant material of tame hay plots, as fodder forages for beef cattle, and the potential for tannin-altered nutrient availability in stockpiled beef cattle manure. As 'plants tend to produce complex mixtures of tannins, and not all tannins have the same effects' (Mueller-Harvey 2006), expected values are expressed as a range, rather than a concrete figure. This reflects the need for thorough and concise isolation and characterization of plant phenolics as described by Haslam and Gupta (1978), whereupon genotypic-specific substances can be identified and named. Such a system would remove much of the ambiguity in nomenclature of plant polyphenol research, while allowing for more accurate quantitative comparisons in research across time and place.

2.2 Tannin Chemistry

Through the lens of a chemist or biochemist, it is understandable how the sweeping characterization of such a broad range of compounds into a single, colloquially-derived term - *tannin* - undermines their complexity and specificity (Hulse 1980). In systems-level ecological and agricultural research, such specificity in characterization and nomenclature is secondary in importance to functional biological influence. Haslam

(1988) eloquently challenged this view in saying "If tannins are so generalised in their action as supposed, it is very difficult to rationalise this generality with the very structural diversity found amongst the plethora of plant polyphenols". This review attempts to reconcile these diverse vantage points, while highlighting the imperative for concurrent, multi-disciplinary research which addresses the specific characterization of tannins and their roles in general ecosystem function.

2.2.1 Definitions

Tannins are phenolic ligands which are prone to forming soluble or insoluble complexes with other molecular polymers, such as proteins, lignin, and polysaccharides. (Zucker 1983; McManus et al. 1985). Produced in a wide range of trees, forbs, herbs, grasses, and legumes, they are the fourth most abundant terrestrial biochemical product, behind cellulose, hemicellulose, and lignin (Hernes and Hedges 2004). In spite of this proliferation, a comprehensive understanding of the functional roles of tannins, from a molecular to an ecosystem level, is sorely lacking. Tannins are differentiated from other polyphenols, including another recalcitrant structural component, lignin (Stafford 1988), and non-tannin phenolics by their bioactive properties.

An affinity for binding has played a central role in defining and characterizing tannins at all levels of research. As such, Bate-Smith and Swain (1962) arrived at an early definition of tannins as being "Water soluble phenolic compounds having molecular weights between 500 and 3000 (Daltons) and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin and other proteins".

Although a more general definition than that of Bate-Smith and Swain (1962) was proposed by Horvath (1981), the five criteria listed by Haslam (1996) for distinguishing

polyphenols with tannin activity reflects the broadest span of observations in biochemical, agricultural, and ecological research over the past 35 years, and are as follows:

i. Some degree of water solubility, facilitating polyphenol-polyphenol interactions.
ii. Molecular weights between 500 and 3000-4000.
iii. A structure of 12-16 phenolic groups and 5-7 aromatic rings per 1000D mass.
iv. Intermolecular complexation or the affinity for protein and polysaccharide binding, mineral chelation, and alkaloid precipitation.
v. Structural characteristics of condensed tannins, hydrolysable tannins, or phlorotannins.

Hydrolysable tannins are are characterized by multiple esterification by phenolic acids of the hydroxyl groups of a predominantly β-D-glucose nucleating agent. Esterification of the carbohydrate by gallic acid yields gallotannins (Mingshu et al. 2006), and by hexahydrooxydiphenic (egallic) acid, ellagitannins (Salminen et al. 2001). Hydrolysable tannins are lower in molecular weight and are more easily cleaved from their host tissues via pH-dependent hydrolysis than larger, flavanol-based condensed tannins. Hydrolysable tannins and their constituent components upon degradation exert less influence on tannin-mediated processes than the larger, more complex condensed tannins (Nierop et al. 2006b). Phlorotannins are a relatively minor class of polyphenols present only in certain aquatic algal species (Haslam 1996). Given that the present study addresses known condensed tannin-producing species in a terrestrial agricultural ecosystem, this review will focus on the remaining form within classical tannin classification (i.e. Freudenberg 1920), condensed tannins.

2.2.2 Condensed tannin structure

Condensed tannins, otherwise commonly referred to as proanthocyanidins, or leucoanthocyanidins in early literature, are polymeric flavan-3-ols or occasionally flavan-3,4-diols (Leinmuller et al. 1991). They are linked by C-C bonds, primarily at the C-4 to C-8 positions (Balas and Vercauteren 1994), though C-4 to C-6 linkages exist in branched polymers (Clifford and Scalbert 2000). Depending on the hydroxylation pattern on the B ring, these linkages lead to either procyanidin or prodelphinidin dimers (Nierop et al. 2006a); the more stable and insoluble condensed tannins invariably exist as polymeric or oligomeric chains in mixtures of procyanidin and prodelphinidin, with branching occurring via C-4 to C-6 linkages or double substitution on the A ring (Porter 1988). The most widely distributed constituent phenolic flavan-3-ol monomers are catechin and epicatechin (Figure 2-1). Other moieties, such as gallocatechin and epigallocatechin, afzelechin and epiafzelechin, or phloroglucinol exist in lesser frequency (Porter et al. 1986; Haslam 1988; Haslam 1998).

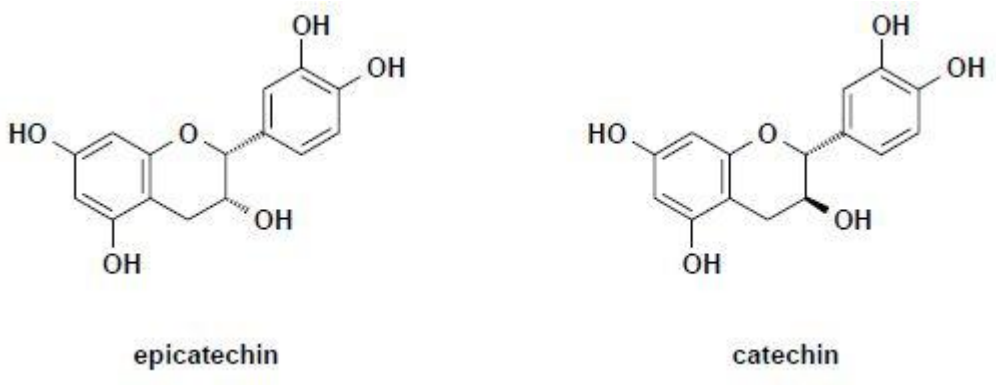
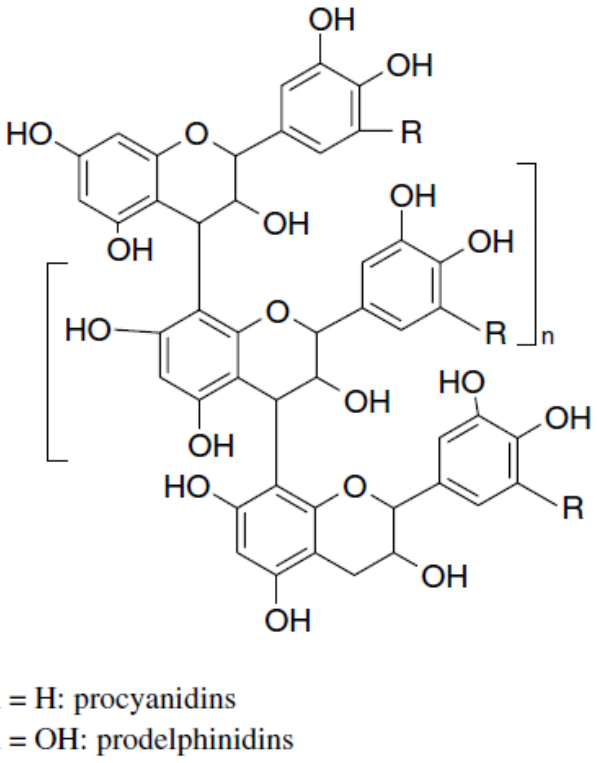


Figure 2-1: Common condensed tannin monomers – epicatechin and catechin (from Hagerman 2002).

Beyond these basic foundations, branching pattern, substitution, chain length, monomeric composition (Figure 2-2), and inter-flavan stereochemistry of condensed tannins vary widely between species (Jones et al. 1976; Kraus et al. 2003), within a single

species (Koupai-Abyazani et al. 1993a), and even within a species' accession (Regos et al. 2009) over a growing season (Lees et al. 1995; Gebrehiwot et al. 2002).



R = H: procyanidins
R = OH: prodelphinidins

Figure 2-2: Example of a sainfoin condensed tannin polymerization (from Mueller-Harvey 2006).

The remarkable complexity and variability revealed through condensed tannin characterization over the past thirty years underpins the difficulty of achieving synergy between quantitative chemical and qualitative biological tannin research.

2.2.3 Tannin synthesis

Tannins are widely distributed secondary plant metabolites found in all major groups of gymnosperms and most groups of angiosperms. Their presence in the most primitive gymnosperms and ferns indicates that their introduction closely mirrored the

development of vascular plants, in a manner similar to lignin (Hernes and Hedges 2004). They are produced in woody structural tissues (Stafford 1988), stems (Aerts et al. 1999), leaves (Jackson and Barry 1996; Regos et al. 2009), reproductive tissues (Jones et al. 1976; Terrill et al. 1992; Morris et al. 1993), and roots, and the only common denominator across plant types and species is that partitioning amongst these tissues is highly variable and genotypic-specific.

The synthesis and allocation of tannins at a cellular level has received little attention in the literature, although it is commonly assumed that tannins are contained within vacuoles, and imbedded within cell walls (Haslam 1996). Lees et al. (1995) established that in *O. viciifolia* leaves, condensed tannins are first present in vacuoles of abaxial subepidermal leaves prior to unfolding, which are subsequently filled during maturation. Thereafter tannins are translocated to adaxial subepidermal vacuoles before being catabolised prior to senescence.

Partitioning tannins into storage vacuoles serves two potential purposes. By tightly controlling tannins at a cellular level, the potential for inadvertent plant auto-toxicity is minimized (Grundhöfer et al 2001). Only upon tissue maceration would their astringent properties within both the plant's histological compounds and the animal become active (McMahon et al. 2000; Waghorn and McNabb 2003). Similarly, partitioning condensed tannins to reproductive tissues as a pathogen or herbivory defense mechanism has been proposed (Terrill et al. 1992; Iason et al. 1995).

Plant production of condensed tannins is thought be a genetically and environmentally mediated extension of the flavonoid biosynthesis pathway (McMahon et al 2000), which produces the component flavan-3-ols of catechin and gallocatechin (Hemingway and Foo 1983; Stafford and Lester 1985). As explained in Haslam (1998), the biosynthesis of polymeric and oligomeric condensed tannins is still somewhat of a

mystery. Haslam (1998) speculated that the process *in vivo* could be mediated by either permissive enzymes, or, as in the case of very insoluble condensed tannins with carbohydrate moieties, by encounters of flavan-3-ol monomers with highly reactive quinone methide intermediates. The resultant variability in condensed tannin structural and chemical profiles mitigates their use in plant species characterisation (Koupai-Abyazani et al. 1993a).

2.3 Tannins in forest ecosystems: what we can learn from inter-disciplinary study

This section reviews the body of literature on tannins in natural ecosystems. Upon review of tannin literature, it appears that previous agricultural research has rarely drawn from natural systems to explain tannin impacts in forages, ruminants, and their associated agricultural ecosystems. This demarcated approach to tannin study may be reconciled by studying the analogous microbiological, soil, and plant processes between each discipline.

The significant energetic costs of tannin synthesis in non-structural plant tissues was long believed to be justified as a response to avian, mammalian, or insect herbivory. The herbivory and pathogen deterrent hypothesis of tannin production, though well-established, has been questioned in some studies (Bernays et al 1989; Schultz 1989; Close and McArthur 2002). For the high energetic cost to a plant in producing tannins – complex polyphenolic structures – the comparative payoff in terms of acute toxicity to predators is very low in comparison to alternative chemical defences and deterrents (Rosenthal and Janzen 1979; Hartmann 2004). These two inconsistencies, coupled with the aforementioned extraordinary variability in tannin forms and concentrations between and within species and environments, have led researchers to postulate that the functional

roles of tannins extend beyond plant-herbivore or plant-pathogen interactions, and even well beyond the physical structure of the plant itself.

Environments dominated by tannin-rich plants and litter layers are often, in an ecological sense, polar to highly productive, uncompetitive, and rapidly-cycling agricultural systems in that they are often nutrient-limited (Northup et al. 1998) and subject to extreme temperature or drought stress (Fierer et al. 2001). However, by studying tannin-producing plants and these native environments, one can develop a more informed framework to interpret how and why tannins in domesticated forages are produced, what their influences will be in a production scenario, and eventually, how they can be effectively exploited in productive and sustainable agricultural systems.

2.4 Tannin influences in forest litter layers and soils

Tannins exist in natural systems as three distinct pools of live and degraded organic matter:

1. Above ground, in live or senescing foliar plant parts.
2. At ground level, as a component of senesced foliar material and other plant detritus or leachate from these materials, accumulated in an organic litter layer.
3. Below ground, as live and degraded root tissue, in humified organic matter, as sorbed components of organic or mineral soils, and as root exudates.

Beyond their production and polymerization in live plant tissues, as illustrated in the figure below, tannins are vulnerable to a host of chemical and metabolic processes which degrade or alter their structure, and ultimately, their biological activity (Figure 2-3). Results of these processes, as relevant to tannins in domesticated forages, will be briefly surveyed here.

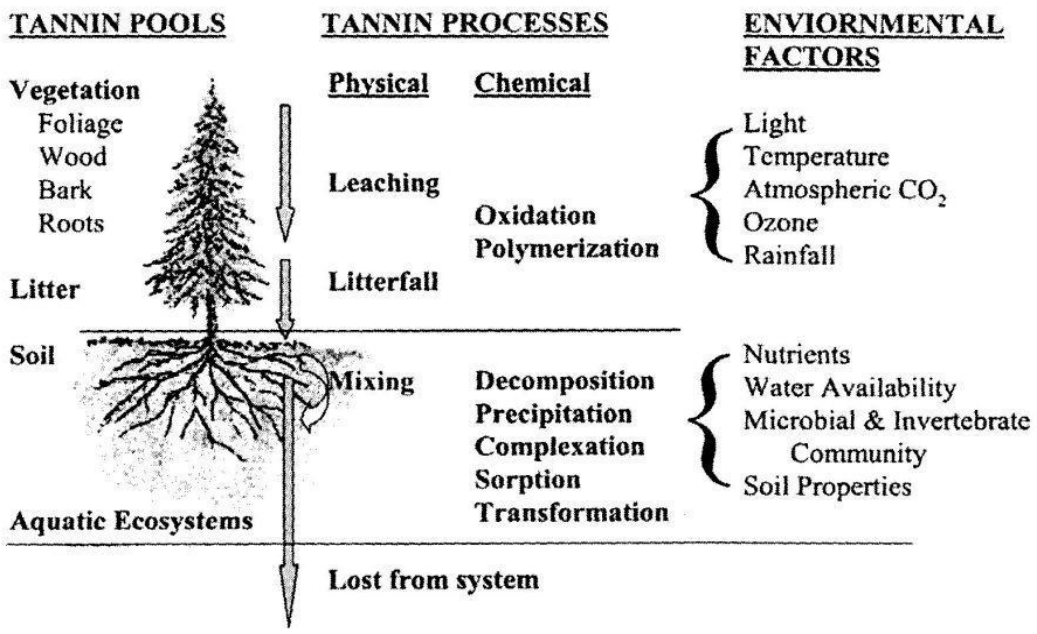


Figure 2-3: Tannin pools, processes, and environmental factors affecting their production and fate in forest ecosystems (from Kraus et al. 2003, modified from Tiarks et al. 1989).

2.4.1 Microbial activity and enzyme inhibition in litter and soils

Tannins are generally thought to act as inhibitors of general soil microbial activity (Swain 1979; Baldwin et al. 1983), and this activity is highly dependent on both the molecular weight of polyphenolic compounds (Lewis and Starkey 1968; Baldwin et al. 1983; Bhat et al. 1998), and soil nutrient status (Fierer et al. 2001). Many groups of soil microorganisms readily use tannins as an energy source (Grant 1976; Scalbert 1991). Variable responses to tannin amendments are reflective of a soil's indigenous microbial community structure, with emphasis on adaptation or selection for microbial polyphenolic metabolism (Cowley and Whittingham 1961).

Evidence of the mechanisms of microbial inhibition by tannins is varied as studies have shown that it can be caused by direct toxicity (Field and Lettinga 1992), co-factor

deprivation (McDonald et al. 1996), or extracellular enzyme inactivation (Benoit and Starkey 1968). Fierier et al. (2001) demonstrated that lower molecular weight tannin additions (<1000D) to organic soils were readily degraded as carbon substrates by microorganisms. Higher weight fractions (>1000D) inhibited microbial respiration. This agrees with Kraus et al. (2004) in that early respiratory CO_2 flushes following exogenous tannin application to soils are likely the result of lower weight tannin metabolism, and that decreased respiration over time may be caused by increased tannin sorbtion to soil or organic matter.

2.4.2 Protein precipitation

Protein-binding capacity of plant tannins has been used as an aggregate indicator for a host of interactive processes – microbial inhibition, nutrient cycling, and organic matter decomposition. Research in tannin-protein astringency has persisted as a predictive measure of tannin bioactivity in herbivores and ecosystems (Handley 1961, Lewis and Starkey 1969; Hagerman and Butler 1980, 1981; Martin and Martin 1982; Baldwin et al. 1983; Halvorson and Gonzalez 2006; Rillig et al. 2007; Adamczyk et al. 2008).

Martin and Martin (1982) stated that 'protein-precipitating capacity is a measure not simply of the quantity of tannins present, but rather of a property of the extract which depends upon the quantity and quality of protein-precipitation agents present'. This specificity of interaction between proteins and tannins has been echoed in all other venues of tannin research, as has the difficulty of measuring activity *in vivo*, whether it is in a forest soil or a ruminant gut. Inference of how tannin concentration, species origin, and structural variation impact an ecosystem and its inhabitants hinges upon reliable and easily-standardized quantification and characterization *in vivo* (Mueller-Harvey 2006).

2.4.3 Nitrogen cycling

The effects of tannins upon microbial communities impact nitrogen dynamics through protein sequestration as a consequence of protein-tannin complexes (Handley 1961) that promote nitrogen immobilization, and the use of tannins as a microbial carbon source which also impact carbon to nitrogen ratios. The manner in which tannins increase microbial N immobilization in lieu of organic N mineralization has been studied extensively (Palm and Sanchez 1991; Kalburtji et al. 1999; Kraus et al. 2004a), as has the inhibition of nitrification by direct toxicity or substrate deprivation (Basaraba 1964; Lodhi and Killingbeck 1980; Clein and Schimel 1995; Northup et al. 1998). The combination of these two processes – sequestration (sorbtion) of organic N sources, and limited oxidation of organic-derived ammonium-nitrogen (NH_4^+) - increases synchrony between labile nitrogen supply and plant uptake. This contrasts with domestic agricultural systems where nitrate-nitrogen (NO_3^-) dominates available nitrogen pools. When extrapolated further, as in the case of extremely infertile soils sustaining high-tannin producing plant species, nitrogen cycling may shift towards an organic-dominated pathway (Stevens and Wannop 1986; Northup et al. 1995, 1998), and over an extended period of time, protect and supply a nitrogen source for humus formation in otherwise inhospitable circumstances. The consequence of tannin application via resident plants or purified extracts to agricultural soils has not been studied directly.

2.4.4 Organic matter sequestration and soil building

Tannin influences upon soil microbial activity and nitrogen cycling inevitably has inevitably led researchers to investigate their potential roles in organic matter retention. While observing the soil and litter characteristics of the native environments of tannin-containing plant species, researchers noted that these areas had unusually well-developed layers of undecomposed litter, sometimes accompanied by an organic layer of *mor* humus

above or in place of mineral soils (Kraus et al. 2003). In some instances, these litter accumulations occurred in nutrient-depleted systems lacking proliferous vegetative growth and associated litterfall; these observations dovetailed almost seamlessly into a well-established model of humus formation: the polyphenolic theory.

The basis for the polyphenolic theory of humus formation stems not from a desire to replicate aspects of the lignin theory (Waksman 1932), but from a need to quantify the distinct contribution of soil microorganisms' interactions with polyphenols in soil. In certain ways, it is analogous to the lignin-protein theory of Waksman (1932), taking into account a predominantly aromatic and nitrogenous structure of a theoretical humus molecule (Stevenson 1982; Paul and Clark 1989) (Figure 2-4), formed through multiple pathways. As mentioned by Stevenson (1982), only lignin and microorganism-derived polyphenols have received much attention in research with respect to humus formation, building on the classical work done by Kononova (1966) and Flaig (1975), respectively.

Central to the theory is the formation and activity of quinones, an intermediary in tannin biosynthesis. These phenol-derived hydrocarbons result from either lignin decomposition or microbial synthesis (via a shikimic acid pathway) (Stafford 1988). This process in a natural environment is generally agreed to be both aerobic and enzymatically mediated, where phenolase and laccase enzymes differentially attack polyphenolic compounds (Schubert 1965). Upon formation, quinones can act as free radicals, either condensing into complex structures without addition of extraneous material or more likely, incorporating amino acids and peptides. The resulting structure has a portion of these molecules attached as amino side-chains, while others become implicitly fixed within the aromatic polymer (Stevenson 1994; Halvorson and Gonzalez 2008). Recently, a paradigmatic shift away from the theory of macromolecular polymers has been proposed by Piccolo (2001) in which a supramolecular structure applied to

humic substances. This theoretical humus structure is bound by pH-dependent hydrophobic, dispersive forces.

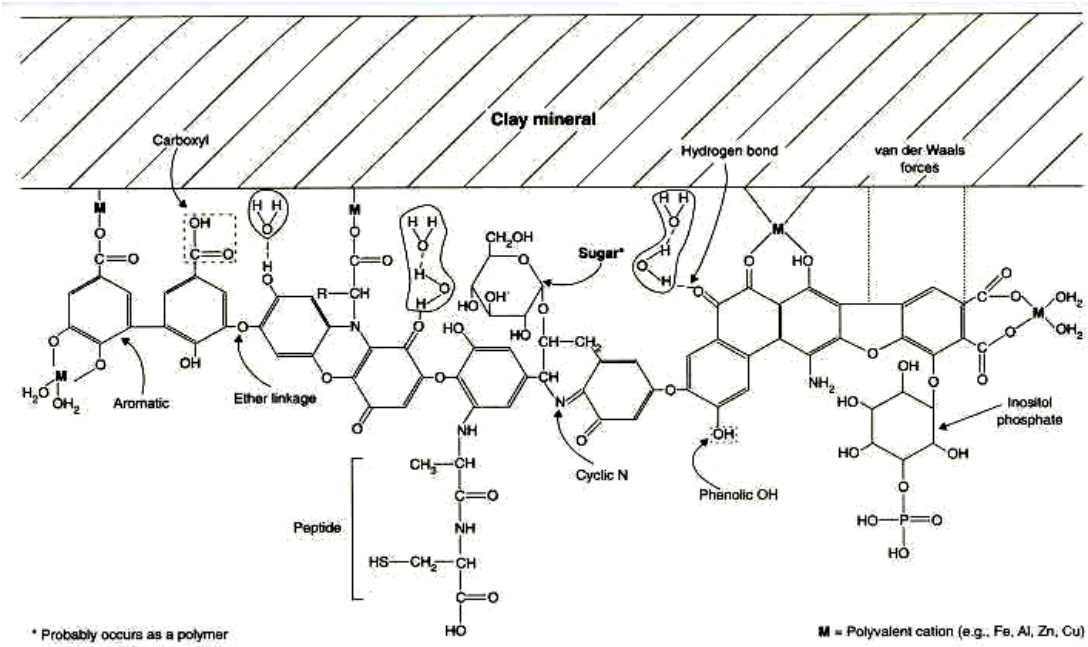


Figure 2-4: A hypothetical structure of humus, forming a complex with a clay particle (from Paul and Clark 1989).

Little work has been done to assimilate the concepts of polyphenolic metabolism into this new model, for the simple reason that identifiable tannins have rarely been extracted from mineral soils (Schofield et al. 1998), and when successful, recovery has been exceedingly low (Rice and Pancholy 1973). However, since both the polyphenolic theory and supramolecular structure of humic substances both involve the presence of recalcitrant polymers of branched aromatic structures held together by hydrophobic bonds, it is very likely that when present in litter and root masses, condensed tannins and their derivatives play a crucial role in humus formation.

2.4.5 Other tannin effects

Tannins have been implicated in other facets of natural systems research, such as ecological succession (Northup et al 1995, 1998; Schimel et al. 1996; Joanisse et al. 2007) as a result of these aforementioned effects on nutrient cycling and allelopathy (Suoto et al 2000). As described by Kraus et al. (2003), tannins may also serve to complex with metals such as Fe, thereby resulting in Fe deprivation for microbes (Scalbert et al. 1999), amelioration of Al toxicity in acid soils (Powell and Rate 1987, Kraal et al. 2009), and increased cation availability via complexation (Slabbert 1992).

2.5 Impacts of tannins in ruminant livestock diets

Similar to their myriad of effects upon an ecosystem, tannins can play a crucial role in the feed intake, health, and nutrition of ruminant animals feeding upon tannin-containing grasses, legumes, and other browse. This section will briefly survey the mechanisms behind tannin-ruminant interactions, and relate these interactions to sustainable livestock production systems.

2.5.1 Tannin pathology

Though their study in agricultural disciplines is motivated by positive production benefits, condensed tannins have the potential to negatively influence ruminant health. When condensed tannins are fed at levels exceeding natural occurrence in forages or browse (typically >10% forage DM), negative animal effects have been noted, such as depressed feed intake, anorexia, and mucous-covered faeces (Reed 1995). However, these effects were thought to likely be the result of malnutrition and protein deprivation due to slow digestive passage rates and low nutrient availability (Hervas et al. 2003).

Pathological symptoms, such as ulcerative lesions in the rumen and reticulum, have been observed in sheep with intraruminally-dosing of with quebracho tannin extract at a rate of 3g/kg live weight (Hervas et al. 2003). Gut surface irritation rather than tannin absorption was the proposed cause of intestinal damage. Lower doses in the same trial and by Frutos et al. (2000) did not elicit similar pathological or histological symptoms. We can conclude that only when a ruminant is forced to ingest high-tannin forages while already consuming a low-quality, low-protein, and/or high-fibre diet will it exhibit symptoms of malnutrition.

2.5.2 Tannin-protein complexation

As mentioned described above, condensed tannins in natural ecosystems are widely regarded as inhibitory to microbial communities and their subsequent effects upon nutrient cycling, nitrogen availability, and degradation of organic substrates in soil. Not surprisingly, tannins exert similar effects upon ruminal microbial communities. A defining characteristic of plant tannins is their affinity for protein binding. This bonding is primarily hydrophobic and pH-dependent (Hagerman and Butler 1980), though the relative importance of each is unknown. Strength of protein bonding can vary by several magnitudes as strength and specificity of the interaction between proteins and tannins is a function of size, conformation, and charge of the both the protein molecule and the tannin polymer (Hagerman and Butler 1981).

The main effect of tannins on proteins in animal diets is based upon reversible pH-dependent hydrogen bonding, which is quickly initiated upon plant tissue maceration (Waghorn 2008). Tannin-protein complexes are relatively stable at approximately pH 3.5-8.0, and thus conserve soluble plant proteins from microbial and enzymatic deamination in the rumen at pH 6.0-7.0. In general, this leads to lower production of ammonia nitrogen in the rumen and an increased flow of crude protein to the small

intestine (Min et al. 2003). Passage through the abomasum (pH 2.5-3.0) lowers the pH of digesta below the threshold of protein-tannin complexes and tannin-bound protein dissociates, becoming available for potential degradation and absorption in the small intestine (Waghorn et al. 1987). Any improvements in ruminant productivity as a result of being fed tannin-containing diets are associated with increased amino acid absorption and a higher efficiency of feed utilization (Burke 2004; Waghorn 2008).

The concept of pH-dependent condensed tannin activity in ruminant metabolism is supported by numerous studies involving sheep and goats (Waghorn et al. 1987; Barry et al. 1996; Wang et al. 1996b; Min et al. 2001). These studies have found positive impacts on production when dietary tannin content is between 2-5% of forage dry matter (Waghorn et al. 1987; Wang et al. 1996a). Above these levels, condensed tannins are liable to induce protein deficiencies due to inhibitory tannin-protein complexation, and depressed voluntary feed intake (VFI) slower passage rates of digesta from inhibited microbial fermentation (Waghorn et al. 1994). It must be noted that the preceding assumptions of pH-mediated tannin activity have largely been informed through studies using a very small number of species – namely, *Lotus* spp. and sulla. Tannins of other species likely will exhibit different stoichiometric habits due to differing molecular weights, structures, and branching patterns, and thus, will likely behave differently in the digestive tract of ruminant livestock (Mueller-Harvey 2006).

There are unexplored aspects of the impact of tannin on animal metabolism. One such effect is complete tannin-protein dissociation, a phenomenon that has only been observed *in vitro* (Hagerman and Robins 1987). Assuming this does occur *in vivo*; the dissociated free tannin fraction would readily bind to alternate substrates shortly after leaving the abomasum for the duodenum, where pH increases above 5 within one metre of the pylorus (Terrill et. al 1994; Waghorn 2008). Compounds for complexation in the small intestine, as mentioned previously, could include other macromolecules, minerals,

digestive enzymes, partially degraded amino acids, or the intestinal wall itself (Terrill et al. 1994; Waghorn 2008).

Available evidence shows that condensed tannin polymers are not degraded in the rumen, and any further metabolism in the small intestine is unlikely. Recovery and quantification of tannin fractions *in vivo* has been hampered by a lack of reliable and accurate extraction techniques in digesta, feces, and urine. Terrill et al. (1994) noted that although laboratory tannin recovery or identification may not be 100% throughout the digestive tract, it is very likely that conformational changes and complexation with other molecules causes tannin fractions to be undetectable by current methods.

2.5.3 Tannin effects on metabolism – ruminant microbial interactions

From a productivity standpoint, tanniniferous legumes ideally should protect a maximum amount of dietary nitrogen from ruminal proteolytic bacteria (Kopecny and Wallace 1982), increasing the amount of protein for hydrolysis and absorption in the abomasum. Simultaneously, the ideal tannin-containing forage would exert the least inhibitory effect upon fibre degradation by cellulolytic organisms (McAllister et al. 2005).

In vitro studies show that tannin-containing forages can increase rumen undegradeable protein threefold over alfalfa (Broderick and Albrecht 1997). Jones et al. (1994) established *in vitro* that purified condensed tannins from sainfoin effectively inhibited growth and proteolytic activity of *Butyrivibrio fibrisolvens* and *Streptococcus bovis*, a known protagonist in bloat (Cheng et al. 1976) by binding to and likely infiltrating the bacterial cell wall. Conversely, other common ruminal bacteria such as *Prevotella ruminicola* and *Streptococcus caprinus* exhibit protective mechanisms against metabolic inhibition from tannins, or can function while in complex with tannins (Jones

et al. 1994; Cheng et al. 1998). Variation in tannin structures between plant species and variation within ruminal microbial communities mitigates the possibility for sweeping generalizations about tannin-microbial interactions.

Tannins can reduce digestibility of insoluble carbohydrates by two mechanisms: substrate complexation, or direct inhibition of cellulolytic microorganisms as explored in the following studies. McSweeney et al. (1998) fed sheep a diet including 30% *Callandria calothyrsus* and found that populations of cellolytic bacteria *Fibrobacter succinogenes* and *Ruminococcus* spp. were reduced. This agrees with earlier *in vitro* work by Bae et al. (1993) with birdsfoot trefoil tannins, where extracellular endoglucanases were inhibited at higher doses of purified tannin (>400 μg ml^{-1}). A survey of nine tannin-containing forage species by McAllister et al. (2005) showed that digestion of filter paper by *F. succinogenes* in the presence of purified condensed tannins varied from 19.8% (*O. viciifoilia*) to 92.4% (*Coronilla varia*). This activity was not correlated to results from simultaneous studies on tannins' chromophore formation in colorimetric assays, two common protein precipitation techniques, or molecular weight. This lack of correlation between laboratory measures of tannin activity undermines the ability to use one tannin measurement (for instance, molecular weight) as a predictive value for other biological activity (i.e. tannin-protein complexation or fibre degradation). McAllister et al. (2005) concluded that given available methodology and its inherent limitations, *O. viciifoilia* appeared to contain the optimal tannin profile to achieve protein precipitation (to promote rumen undegradeable protein) while not inhibiting cellulose (fibre) digestion.

2.6 Productivity benefits of tannins in ruminant diets

2.6.1 Forage intake, liveweight gain, reproduction, and wool production

Karnezos et al (1994) conducted a metabolism study using lambs in a grazing system, with pasture species of alfalfa, sainfoin, and sainfoin in a mixture with wheatgrass (*Thinopyron intermedium* subsp. *barbulatum*). They found that over two seasons, grazing a sainfoin monoculture resulted in a slightly higher live weight gain (LWG), which was attributed to a 14% higher intake and 20% greater biomass utilization compared to alfalfa. Both the increased intake and biomass utilization were thought to be related to palatability derived from higher concentrations of soluble sugars versus alfalfa. In another study by Douglas et al. (1995), lambs grazing the tannin-containing legume *Lotus corniculatus* (Birdsfoot Trefoil) resulted in a realized 30% greater daily LWG compared to lambs grazing alfalfa. Given that alfalfa and birdsfoot trefoil had similar nutritive profiles, increased productivity was attributed to a higher voluntary feed intake, higher digestibility, and improved utilization of digested nutrients.

More recent work on direct productivity effects of tannins has focused on commercially-prepared tannin extracts as feed additives. Min et al. (2006) found that 2% supplementation of quebracho extract, administered by rumen cannulae in beef steers grazing winter wheat, resulted in a 15% increase in average daily gain (ADG) over a non-tannin containing control. Similar results were reported by Al-Dobaib (2009), who reported significant increases in ADG in sheep fed quebracho tannins at 2% DM in pelleted alfalfa. Reasons put forth for these increases centered on improved protein utilization (Al-Dobaib 2009) and decreased bloat (Min et al. 2006).

Differentiating between tannin-specific and species-specific effects is achieved in ruminant feeding trials through addition of polyethylene glycol (PEG) to tannin-containing forages through water, oral drenches, or as a free-choice supplement

(Waghorn 2008). This feed additive creates stable complexes with tannins, rendering them chemically inactive, thus allowing for intra-specific comparison of tannin containing forages, with and without tannin activity. Results of such studies have varied by species. Condensed tannin in *Lotus corniculatus* have been unanimously positive on ovulation rate (Ramirez-Restrepo et al. 2005), lamb weight gain (Douglas et al. 1999), and wool production (Min et al. 2001) in sheep, whereas slightly negative effects were observed when lambs were fed sulla (Douglas et al. 1999). No comparable studies have been conducted on sainfoin in beef cattle, and elucidation of general positive tannin-specific effects on productivity are circumspect until similar studies are conducted across a broader range of livestock, forage species, and management systems.

2.6.2 Pasture bloat prevention

Tannins have long been known to reduce the potential for frothy or pasture bloat. This potentially fatal condition occurs when gaseous products of fermentation are trapped in a stable foam produced by soluble proteins and simple sugars (Cheng et al. 1998; MacKown et al. 2008). Bloat potential is greatest when grazing young, lush legume forages containing high protein and soluble sugar levels, such as those found in alfalfa and red clover (Majak et al. 2003). Condensed tannins complex labile rumen proteins, slow digestion, and inhibit and collapse the formation of proteinaceous foam (Tanner et al. 1995).

Early work by Kendall (1964, 1966) examining bloat-safe leguminous forages hypothesized that tannins and other phenolics were the bloat-reducing agent. These conclusions were drawn without the benefit of appropriate methods for tannin extraction or quantification. A related survey of 21 tropical legumes by Jones and Lyttleton (1971) reported that all bloat-safe forages showed the presence of "protein precipitants". This preliminary evidence of condensed tannins as bloat reducing agents was later confirmed

by Jones and Mangan (1977), who paved the way for *in vivo* and *in vitro* experiments identifying relative effects of different tannin-containing species on bloat. A study by McMahon et al. (1999) found that soluble protein levels in the rumen were significantly reduced with the addition of CT-containing forage. This complemented previous works showing that the condensed tannin effects from *Lotus pedunculatus* increased non-ammonia nitrogen flow to the duodenum in sheep via tannin-protein complexation (Barry and Duncan 1984; Barry et al. 1986).

In an effort to further quantify the role of condensed tannins in bloat prevention, Li et al. (1996) developed a sensitive chemical technique for quantifying low levels of condensed tannin in plants. By assimilating a range of literature values from tannin-containing feeding trials with their novel screening procedure using 23 legume species, they concluded that the threshold for bloat safety in forage legumes was between 0.1% and 0.5% DM. McMahon et al. (1999) examined this recommendation in a four-year trial on beef steers, where relatively small amounts (10-20% when standardized to dry matter) of sainfoin were added to a fresh-cut alfalfa diet. In three out of four years, addition of sainfoin significantly ($P<0.001$) decreased animal days of bloat by 200-1500% compared to a pure alfalfa diet. They concluded that the dietary condensed tannin concentration of 0.35-0.46% DM provided in a fresh, mechanically-mixed 10% sainfoin/90% alfalfa ration significantly influenced tannin-protein interactions in the rumen, and increased rumen clearance of bloat-inducing proteinaceous foam. Wang et al. (2006) found that grazing sainfoin-alfalfa mixtures with beef steers did not reduce incidence of bloat as much as in confined feeding, and attributed this drop in efficacy to behavioural selection while grazing, and preferential grazing of the more upright alfalfa component of the sward upon re-growth.

There is a renewed interest in effects of tannins on bloat. Recently, research has examined the effects of low levels of endogenous plant tannin production in annual cereal

crop grazing (Mackown et al. 2008) and dietary supplementation of with commercially-prepared tannin extracts (Min et al. 2006).

2.6.3 Reduction in greenhouse gas production and enteric emissions

Modern agricultural practices have been shown to be moderate contributors of nitrous oxide (NO_2) and methane (CH_4) emissions to Canada's greenhouse gas (GHG) budget. Enteric fermentation and manure management produced 23 000 and 7 800 Kt CO_2 equivalents year $^{-1}$, or 38% and 13% of total agricultural emissions according to Canada's 2007 Greenhouse Gas Inventory (Environment Canada 2007). Globally, agricultural GHG production accounted for 10-12% of total anthropogenic GHG emissions in 2005, of which CH_4 contributed approximately 54%, or 3.3 $GtCO_2$-eq/yr. (Smith et al. 2007).

Livestock management systems have been shown to play a significant role in reducing anthropogenic GHG impact (Ominski et al. 2007) through two intimately-coupled avenues: 1) by potentially reducing enteric GHG emissions through ruminant feeding strategies, which 2) incorporate extensive integration of carbon-sequestering perennial forages (Follett et al. 2001; Soussana et al. 2004; Smith et al. 2007; Soussana et al. 2009). Research has thus aimed at simultaneously increasing productivity and decreasing enteric and manure GHG emissions of forage-based livestock production (Johnson and Johnson 2000) while preferentially developing grazing-based feeding strategies over those requiring cut herbage and confined housing (Soussana et al. 2009). Whole-farm approaches to modelling GHG mitigation strategies have found that integration of these "best practices" is region-specific (Stewart et al. 2009).

As with most other ruminant-tannin interactions, research has shown mixed results in the ability of CT's to reduce enteric CH_4 emissions. It has been demonstrated *in*

vitro that both hydrolysable (Field and Lettinga 1987) and condensed (Field et al. 1988; Tavendale et al. 2005) tannins are directly toxic to methanogens. An alternate mechanism behind this inhibition is H_2 deprivation caused by decreased fibre degradation (Carulla et al. 2005).

Several recent *in vivo* experiments have shown that feeding tannin-containing forages to sheep (Waghorn et al. 2002), goats (Puchala et al. 2005), and grazing dairy cows (Grainger et al. 2009) reduced enteric methane emissions by 13 to 30%. Interactions between methanogens and condensed tannins is likely species-specific to both forage and livestock. This specificity has been demonstrated by Beauchemin et al. (2007), who found that quebracho supplementation in a barley silage-based diet did not reduce enteric methane emissions in beef heifers. Conversely, Bouchard (unpublished) found that feeding sainfoin hay to beef steers significantly (<0.05) reduced methane emissions on an absolute (L day^{-1}) and bodyweight (L kg BW^{-1}) basis over alfalfa hay, but not when methane was expressed as a %GEI. Beyond the animal, dietary tannins may additionally reduce NO_2 emissions from stockpiled manure (Misselbrook et al. 2005). Grainger et al. (2009) postulated that adoption of tannin-containing forages for enteric and manure-based GHG reduction would only occur without a penalty in productivity, or if the environmental benefit of their use was valued in monetary terms.

2.6.4 Pathogen suppression

An emerging body of work has focused on the antimicrobial activity of tannins as it relates to native food-borne bacterial pathogen suppression livestock production systems (Krueger et al. 2010). The human health, economic, and management-related costs of pathogenic strains of *Escherichia coli* which have motivated this research have been reviewed by Callaway et al. (2003). Favourable results in *in vitro* study of *E. coli*

survival in bovine feces have been reported after inoculation with tannins derived from aquatic (Wang et al. 2009) and terrestrial (Wells et al. 2005) sources, under simulated Canadian climatic conditions (Berard et al. 2009). This work has been reinforced by a novel *in vivo* study by Berard et al. (2009) where sainfoin forage diets reduced generic *E. coli* numbers in fresh bovine feces. Dietary supplementation of tannins as bactericidal agents in commercial feedlot production was shown to be viable by Kreuger et al. (2010), who demonstrated that supplementation of commercially-prepared mimosa (*Acacia mearnsii*) condensed tannin at 14.9 mg/kg in a corn finishing ration did not negatively impact feed efficiency or animal performance. Further research is necessary to assess potential source and dose-specific efficacy of purified tannins on pathogen survival in a broader range of production scenarios.

2.6.5 Parasite control

Gastrointestinal parasites are a major problem in grazing livestock (Waller 2006), as they reduce liveweight gain by depressing voluntary feed intake, reducing dietary digestion and absorption, and feed efficiency (Hoskin et al. 2000). Chemical control of these internal parasites has traditionally been via broad-spectrum anthelmintic drugs, applied as drenches, boluses, pastes, injections, pour-ons, and in mineral mixtures. These drugs have progressively decreased in efficacy worldwide as a sole method of parasite control, and only one new mode of action drug has been developed in the past 25 years (Jackson and Coop 2000). Combined with increased consumer concern over potential residues of these pesticides in meat and milk products, there is a strong interest in developing non-chemical strategies to combat ruminant parasitism.

Condensed tannin-containing forage legumes have been shown to reduce parasite burdens in both immature and adult sheep and goats. Niezen et al. (1995) used lambs carrying a high parasite load grazing tannin-containing sulla (*Hedysarum coronarium*) with alfalfa. The sulla pasture treatment significantly reduced faecal egg count numbers, and increased liveweight gain by 50% after 28 days compared to lambs grazing alfalfa. In a second experiment involving a lower parasite burden, sulla eliminated parasite-induced anorexia and decreased worm count after 42 days. Similar results have been reported in sheep by Robertson et al. (1995), and in young red deer (*Cervus elaphus*) by Hoskin et al. (2000). Moore et al. (2008) later established that feeding *Sericia lespedeza* (Chinese bush clover) containing tannin concentrations of 87-181 g/kg to young male goats reduced abomasmal worm counts by 37% over non-tannin containing bermudagrass (*Cynodon dactylon*). These *in vivo* studies and others have proven that multiple tannin-containing forage species can successfully reduce parasite loads and augment chemical anthelmintic application in ruminant livestock. Research by Molan et al. (2000) and Paolini et al. (2003) suggest that since L3 stage of infective larvae are strongly inhibited by tannins, condensed-tannin containing forages may interrupt parasite life cycles and reduce pasture contamination with infective nematodes.

The mechanism underlying tannins' anthelmintic properties was first proposed to be nutrition-related, where the host response was improved through increased protein and mineral availability of forages. *In vitro* studies, such as those by Barrau et al. (2005) and Brunet et al. (2008) have supported an alternative hypothesis that condensed tannins exert direct toxic effects on parasitic nematodes. Though limited research has been conducted on direct toxic effects, work by Min and Hart (2003) and Bahuaud et al. (2006) suggests that these toxic effects are specific to parasite, livestock, and forage species. Brunet et al. (2008) examined this specificity using extracts from sainfoin, and concluded that tannin structural differences significantly affect interactions with parasite larvae and eggs. This

corroborates with structure-activity hypotheses of tannins in other research disciplines, and highlights the need for thorough tannin characterization by forage species in order gauge their usefulness as anthelmintic controls (Waller 2006).

2.7 Forage species in this experiment

2.7.1 Meadow brome

Meadow bromegrass (*Bromus biebersteinii*) is a cool-season, bunch-type brome species which was registered in Canada in 1980 (Knowles et al. 1993). It is considered a superior pasture species to smooth bromegrass (*Bomus inermis*) due to rapid re-growth following a haying or grazing event, superior longevity, and marginally higher nutritive value at later maturities (Ferdinandez and Coulman 2001). No *Bromus* species have been reported to contain condensed tannins. Annual productivity in pure stands has been reported from 2082 kg/ha in an unfertilized trial in Saskatoon, SK. to 12 114 kg/ha in a heavily-fertilized and irrigated four-year trial at Lacombe, AB. (Knowles et al. 1993).

Integrated research on pasture productivity, persistence, and nutritive quality of meadow brome in mixtures is sparse (Kopp et al. 2004). Including a grass species in a legume pasture balances seasonal yield distribution for summer and late season grazing, while providing a supply of biologically-fixed nitrogen to the grass species. In this respect, Pearen and Baron (1996) showed that in a mixture with alfalfa under frequent (four times per growing season) defoliation, meadow brome out-yielded smooth brome by 9-19%. Holt and Jefferson (1999) similarly concluded that meadow brome compared favourably in yield and competitive ability to three other cool season grasses when sown with alfalfa; furthermore, it was the only mixture to not induce bloat in yearling beef animals. Bloat reduction in the meadow brome-alfalfa mixture was a function of

increased fibre intake, which slowed protein digestion and passage rates beyond that of a pure legume diet. This common effect of grasses was best expressed by meadow brome in the later stages of the experiment due to superior persistence and productivity versus the other grass species. Mean mixture yield over the seven year trial was 3250 kg/ha., with high seasonal variability (780 kg/ha. to 8030 kg/ha.) which was attributed to moisture and temperature differences between years. Similar research investigating meadow brome in mixtures with tannin-producing legumes is nonexistent.

2.7.2 Alfalfa

Alfalfa (*Medicago sativa*) is the most widespread forage legume in Western Canada (Popp 1997) due to its ability to increase both the quality and productivity of tame or improved pastures (Popp et al. 2000). When grazed, these beneficial qualities translate into higher stocking rates (Jones and Sandland 1974) and higher liveweight gains (Campbell 1981; Kilcher 1982).

2.7.3 Sainfoin

Sainfoin (*Onobrychis viciifolia*) is a perennial leguminous forage crop belonging to a genus originating in Western Europe to Asia, and is adapted to many agricultural areas worldwide (Doyle et al. 1984; Goplen et al. 1991; Xu et al. 2006; Pecetti et al. 2009). Long recognized as an extremely palatable, productive forage crop, research has been committed to studying sainfoin as an alternative perennial legume to alfalfa (*Medicago sativa*) in North American livestock production. This interest has been driven mainly by its resistance to alfalfa weevil (Ditterline and Cooper 1975), as well as the novel tannin-related production benefits of bloat safety (McMahon et al. 2000), anthelmintic activity (Athanasiadou et al. 2005), improved protein utilization (Waghorn 2008) and potential for a reduction in enteric methane emissions (Ominski, unpublished).

Widespread adoption of an unconventional forage crop, even one with novel nutritional traits, will not succeed if it carries a substantial yield penalty and/or increased cost of production. Though there is a body of research on the aforementioned tannin-related feeding qualities of sainfoin, these qualities have not propelled widespread cultivation of sainfoin in commercial production, nor have they been comprehensively reviewed across locations and research groups. On this basis, the following pages review the agronomic qualities of sainfoin under a host of management scenarios with respect to establishment, biomass production, response to temperature and drought stress, stand persistence, and forage nutritive quality.

2.7.3.1 Seeding and establishment

Sainfoin has been reported as an easily established crop due a large seed size (Ditterline and Cooper 1975). Similar to alfalfa, sainfoin responds best when sown in the spring at a 1-2 cm depth (Goplen et al. 1991) into a firm, weed-free seedbed which is packed after sowing (Mowrey and Matches 1991). In a study at four southern Canadian prairie locations, Waddington et al. (1986) found that a pre-seed application of trifluralin (545 g a.i./L emulsifiable concentrate) of 1.12 kg/ha significantly increased DM yields over a herbicide-free control in both the year of establishment and in subsequent years, though the effect was extremely variable between sites. Their study reinforced that sainfoin establishment was possible on a range of soil types in Western Canada.

Successful sainfoin establishment is best achieved with minimal competition to developing plants from either weeds or companion crops. In pure sainfoin stands, narrower row spacing is preferred to increase crop competiveness in the establishment year, and to limit inter-row weed invasion (Goplen et al. 1991; Jefferson et al. 1994). High variability in seed size across sainfoin cultivars can contribute to highly variable seedling vigour and biomass production in the year of establishment (Cash and Ditterline

1996), and is not a reliable indicator for productivity potential in subsequent seasons. Early field research on sainfoin was hampered by poor performance of a sainfoin-specific innoculant, and N-deficiency was often mitigated by substantial applications of inorganic N fertilizer (Smoliak and Hanna 1974; Meyer 1975; Walsh et al. 1983; Mowrey and Matches 1991). A sainfoin-specific *Rhizobium* spp. inoculant is now commercially available (Becker-Underwood 2010), and its use is widespread. In later work done at Indian Head, SK, researchers found that even when properly inoculated, inorganic nitrogen fertilizer rates up to 100 kg/ha did not provide an economically feasible return in production in sainfoin (Holt, unpublished). Harvest in the establishment year will significantly reduce production and persistence in following years, and is not recommended even under ideal conditions (Goplen et al. 1991).

2.7.3.2 Biomass production

Sainfoin has a high early-season productivity potential which, when compared to alfalfa, is quickly limited by intolerance to drought stress and inter-crop competition (Bolger and Matches 1990; Kallenbach et al. 1996), and poor re-growth following a mechanical harvest or grazing event limits late-season production (Peel et al. 2004). Smoliak and Hanna (1975) found that sainfoin grazed by sheep under subirrigated conditions yielded more forage biomass than alfalfa in the first of three grazing cycles (3410 kg/ha sainfoin vs. 2876 kg/ha alfalfa) and an equal amount (2545 kg/ha sainfoin vs. 2553 kg/ha) in the second cycle. Similar prolific early season growth has been demonstrated in dryland (Hanna and Smoliak 1968; McGraw and Marten 1986) and irrigated (Ditterline and Cooper 1975) trials. Early season growth combined with near-dormancy during dry summer conditions led Bolger and Matches (1990) to suggest that sainfoin is best adapted as an early season hay or pasture crop where moisture is not a limiting factor, while McGraw and Marten (1986) disputed the value of sainfoin under any growing conditions due to extremely poor regrowth in Minnesota.

Carleton et al. (1968) established that maximal biomass and protein yields were achieved for both sainfoin and alfalfa at approximately the same date on dry land, at 70% and 5% bloom, respectively, and that irrigation extended these values to later bloom stages. Peel et al. (2004) surveyed 13 sainfoin cultivars from international and domestic sources, and found that the highest-yielding sainfoin variety – "Pola" from Turkey – produced 27% less DM than Deseret alfalfa under four irrigation regimens. Significant variation in seasonal growth distribution between sainfoin cultivars was apparent.

In the Canadian prairies, sainfoin in monoculture has been shown to yield between 15-25% less than alfalfa. Goplen et al (1991) noted that over a five-year trial in Lethbridge, AB., sainfoin yields (6310 kg/ha) were 89% lower than those of alfalfa (7110 kg/ha). During the same period, a number of dryland trials comparing Melrose and Eski sainfoin varieties to either Beaver or Ladak Alfalfa showed very high absolute and relative variation between sites and years (Goplen et al. 1991). For instance, over five site years at Swift Current, SK., Melrose sainfoin yielded 1940 kg/ha DM, or 81% of alfalfa (2380 kg/ha) (Goplen et al. 1991). Comparatively, two site years at Winnipeg, MB showed that Melrose sainfoin exceeded alfalfa yield by 5% (Goplen et al. 1991).

Performance of sainfoin in grass-legume or legume-legume mixtures is highly dependent upon seeding method, species selection, and agronomic management. Cooper (1972) found that alternate-row seeding (15cm spacing) of either black medic (*Medicago lupulina*) or birdsfoot trefoil with sainfoin improved 4-year yields over sainfoin monocultures by 2% and 9%, respectively. The significant advantage observed in the trefoil mixture was attributed to increased competitive ability of the sward against dandelion invasion after three years. Jefferson et al. (1994) showed that sainfoin + alfalfa mixtures could occasionally match the yield of pure alfalfa stands, though poor and variable sainfoin persistence in the mixtures could potentially result in unreliable effects on bloat reduction.

Hanna et al. (1977) made comparisons between various grass species in mixtures with alfalfa and sainfoin, and found that legume yield and total yield were higher in alfalfa-grass mixtures, regardless of grass species, seeding method, or year. With nearly identical yields of the grass component, alfalfa-grass mixtures averaged 1180 kg/ha DM more than sainfoin-grass mixtures. Total yields of sainfoin-grass mixtures were not significantly different regardless of the grass species in the mixture. This agrees with Hanna et al. (1977) who showed that under ideal growing conditions, there was no significant yield difference between sainfoin mixtures with crested wheatgrass (*Agropyron desertorum*), Russian wild ryegrass (*Elymus junceus*), or pubescent wheatgrass (*Agropyron trichophorum*). Based upon these criteria, success in growing sainfoin in a mixture is most dependent upon environmental conditions which may select for either species in the mixture.

2.7.3.3 Stand persistence

Perhaps the greatest drawback to sainfoin in commercial forage production is a lack of stand persistence, due to a combination of poor winter hardiness and poor growth in drought conditions. When sown in mixtures, sainfoin may be dominated by competitive legume or grass species including red fescue (*Festuca rubra*) and Kentucky bluegrass (*Poa pratensis*) as described by Cooper (1972), alfalfa as described by Jefferson et al. (1994), and meadow fescue (*Festuca pratensis*) and perennial ryegrass (*Lolium perenne*) as described by Liu et al. (2009). The latter found that even in a 1:2 grass to sainfoin ratio, sainfoin plant populations were reduced to half after two seasons. Cooper (1972) concluded that sainfoin-grass stands were amongst the most competitive swards against perennial weed invasion, however, the competitiveness of theses grasses, as well as white clover (*Trifolium repens*), reduced sainfoin productivity over four years to below that of a sainfoin monoculture. It was noted that in both mixtures and pure stands, the sainfoin cultivar Remont was found to be more competitive than the cultivar

Eski. In a later effort to introduce a tannin-containing forage into alfalfa stands, Jefferson et al. (1994) found that although alternate-row seeding of sainfoin and alfalfa could match yields of monoculture alfalfa, sainfoin was reduced to <10% biomass in two years.

2.7.3.4 Forage nutritive quality

In both grazing and feeding trials, sainfoin has consistently been identified as extremely palatable forage in comparison to other common temperate legumes and grasses (Parker and Moss 1980). Holden (1963) established early that this is likely due to the lower crude protein, higher free nitrogen, and lower crude fibre content than alfalfa. In a study on the effects of harvest date and irrigation on the nutritive value of sainfoin and alfalfa, Carleton et al. (1968) confirmed these findings, adding that sainfoin was also 24% lower in ash and 16% lower in calcium, and 32% higher in P across all stages of maturity when compared with alfalfa. Similar results were reported by Parker and Moss (1980) and Klady et al. (1979,) who showed 26% and 41% lower crude protein in sainfoin when compared with alfalfa. Iwaasa et al. (2006), on the other hand, found proximate analysis of alfalfa and sainfoin from Swift Current, SK. were not considerably different.

A novel study comparing the mineral concentrations in alfalfa and sainfoin under a soil moisture gradient found that moisture did not have an impact on relative mineral concentrations, and that compared with alfalfa, sainfoin had higher concentrations of Mg and P, lower concentrations of Ca, K, and Cu, and a similar Ca:P ratio (Kidambi et al. 1990).

2.7.3.5 Tannin production

The novel qualities of sainfoin – bloat safety, anthelmintic properties, reduced enteric GHG production, and improved nitrogen efficiency in ruminants – are largely the

providence of condensed tannins contained in the leaves and reproductive organs of the plant (Lees et al. 1993). The first direct reference to the tannin content of sainfoin was made by Jones and Lyttleton (1971) while screening for bloat-safe forages in New Zealand. As a follow-up to this protein-precipitation work, Jones et al. (1976) attempted to isolate, purify, and characterize condensed tannins based on molecular weight. They found that sainfoin leaves contained the least astringent tannins of all plant species as a result of a high delphinidin:cyanidin monomeric ratio (81:19).

Plant tannin concentration changes with maturity. Early sainfoin tissue culture work by Lees (1986) demonstrated that tannin-containing cells which formed after 21d in callus explants disappeared after 32d, and that this phenomena was genotype specific. Further characterization of sainfoin tannins was conducted by Koupai-Abyazani et al. (1993a, 1993b) in two concurrent studies. They established that tannin polymeric characteristics (molecular weight, degree of polymerization, stereochemistry) between cultivars and accessions were quite similar, in that polymers showed a high content of prodelphinidin and *cis*-isomer units, and thus could not be used reliably in classification. They noted that despite these similarities, tannin synthesis and polymer formation is a dynamic process and changed during maturation. Lees et al. (1993, 1995) re-affirmed this theory in a histological study of sainfoin tannins during plant development. Using a combination of light and electron microscopy, they found that tannins in sainfoin leaves are subject to synthesis, turnover, and degradation, and speculated that the high energetic investment in tannins may be a hallmark of "luxury" secondary metabolite metabolism, controlled independently of biomass accumulation.

Molecular weight of tannins in sainfoin (*Onobrychis viciifoilia*) has been confirmed by multiple sources (Foo and Porter 1980; Koupai-Abyazani et al. 1993a; Marais et al. 2000). Haslam (1994) re-affirmed this observation by adding that high molecular weight (>17000) metabolites, such as those seen in *O. viciifolia* by Jones et al.

(1976) and Khanbabaee and van Ree (2001), are inappropriately classified as tannins, as they would not exhibit the solubility in aqueous media necessary to facilitate some level of polyphenol-polyphenol interaction in a natural state. These large and insoluble molecules, with a carbohydrate-anchored terminal end (Haslam 1998), are more analogous to lignin than tannins (Stafford 1988), and likely play a greater structural role than a metabolic one.

Thorough deconstructive analysis of sainfoin polyphenols have since been conducted (Lu et al. 2000; Marais et al. 2000; Regos et al. 2009) in an effort to link chemical structure and composition to bioactivity, however, these efforts have not been complemented by in-vivo, whole-plant study of sainfoin tannin production.

Few references are available for whole-plant condensed tannin concentration in sainfoin. Knox et al. (unpublished) found that vegetative sainfoin contained significantly higher tannin concentrations (58.7 g/kg) compared to mature sainfoin (33.7 g/kg). These values can be compared to other Canadian sainfoin hay tannin concentrations observed by McMahon et al. (1999) of 28 g/kg DM. Molecular characterization of condensed tannins, along with relevant biological assays, will be the tools with which plant breeders screen for beneficial tannin content in forages (Mueller-Harvey 2006). Systems-level study of tannin-producing forages under commercial production scenarios are the tools which agronomists and extension agents will measure the efficacy of any laboratory efforts. Such research is notably absent in sainfoin.

In order to draw some conclusions on what, if any, effect that environment, fertility, and competition may have upon tannin production in sainfoin, one currently has to look at similar research conducted with other *Lotus* species. Impact of soil fertility on tannin concentration is species-specific. Using birdsfoot trefoil *(Lotus corniculatus*), Briggs (1990a) found that inorganic nitrogen fertilization modestly increased tannin

content over an N-depleted control. This unexpected result was partially explained in a concurrent study which showed significant (≤ 0.03) and positive correlation between plant dry weight and tannin concentration over the first 12 weeks of growth. Birdsfoot trefoil tannin concentration was previously identified as showing little to no response to fertility status (Lowther et al. 1987). In contrast, a study using big trefoil (*Lotus pedunculatus)* showed that high fertility conditions decreased tannin concentration to 20-30g kg^{-1} from 80-110 g kg^{-1} in acidic and infertile soils (Barry and Forss 1983). Further application of P and S fertilizer to those acidic soils reduced tannin concentrations of *L. pedunculatus* to 40-50g kg^{-1}, which led to the conclusion that adverse environmental conditions induces tannin production (Barry and Duncan 1984). Increased tannin content in both big and birdsfoot trefoils over periods of high temperature and low soil moisture led Anuraga et al. (1993) to conclude that condensed tannin concentrations were elevated primarily in proportion to the size of the reduction in growth rate, a conclusion repeated verbatim in a review by Lascano et al. (2001).

Lastly, interspecific competition may influence tannin concentration. The only study recognizing this phenomenon examined tannin concentration in conventional and rhizomatous birdsfoot trefoil in mixtures with tall fescue (*Festuca arundinacea*) (Wen et al. 2003). Birdsfoot trefoil grown in mixtures had tannin concentrations 55% ($P < 0.05$) and 100% ($P<0.10$) greater than that grown in pure monoculture stands in two years, respectively. It was speculated that changes in light interception, grazing patterns, or microenvironment may have affected condensed tannin concentration. To date, this is the sole example of competitive stress on tannin production in leguminous forages.

2.8 Methods of tannin evaluation

Tannins are a highly diverse group of plant secondary metabolites which are often produced in complex mixtures. This inherent diversity has facilitated the development of a dizzying array of techniques for tannin quantification and characterization since the 1950's; the holy grail of tannin methodology allows inter-specific and quantitative comparison of all tannin-related bioactivity as it pertains to the desired end-use. Such methodology would accommodate inter-disciplinary discussion on tannin functions in both natural systems and sustainable agricultural systems. Given that a contemporary review paper on the subject requires the premonitory words "unravelling" and "conundrum" in the title (Mueller-Harvey 2006), the search for this elusive methodology continues to this day.

2.8.1 Sample preparation and tannin extraction in plant material

Prior to entering a laboratory, the harvest, transport, and drying methods used can significantly influence the accuracy of later extraction and assay procedures (Waterman and Mole 1994). In general, harvested plant material should be kept in the shade, transported from the field as quickly as possible in cool temperatures, and then frozen prior to drying. This ensures that oxidative or light-catalyzed decomposition of phenolics will be limited (Okuda et al. 1989). Mueller-Harvey (2001) stated that fresh plant material was preferable to dried and ground samples, however, it has been speculated that representative tannin recovery to ruminant ingestion requires substantial cellular disruption (Haslam 1988), though it has been shown that freeze-dried and fresh plant material show similar tannin values (Terrill et al. 1990). Drying is logistically preferable to using fresh material, as it frees researchers to conduct time-intensive extraction and assay procedures outside of a growing season. Drying method itself can also significantly alter tannin recovery. Wolfe et al. (2008) compared oven drying (55^{o}C for

48h.) to freeze drying using five native legumes from Texas, and found that oven drying drastically shifted condensed tannins to protein and fibre-bound forms. This was accompanied by a substantial and variable (6 - 521%) reduction in extractable (soluble) condensed tannin concentrations, while often significantly over-stating total condensed tannin concentrations compared to freeze-dried samples. Whether using freeze-dried or oven-dried ground samples, most condensed tannin assays are done on material extracted with either aqueous acetone or methanol (Schofield et al. 2001), though acetone is the more efficient solvent (Terrill et al. 1990).

2.8.2 Tannin characterization and quantification in plant material

There are many methods available for evaluating extracted tannins. The relative value of any tannin methodology is contingent upon it's relevancy to the biological function of the tannin-containing plant under study. For instance, a ruminant digestive physiologist maybe concerned with the protein-precipitating capacity of forage-derived tannins in digesta, where a forest ecologist may be more inclined to study potential inhibitory effects of both hydrolysable and condensed tannins on litter decomposition. The most common tannin assays are chemical colourimetric procedures (Hagerman 2001; Mueller-Harvey 2006). Several other techniques are available for further characterization of tannin reactivity, structure, and conformation, which include protein-precipitation assays (Asquith and Butler 1985), enzymatic assays (Fickel et al. 1999), and high performance liquid chromatography (HPLC) (Cheynier et al. 1999). These, among other techniques, have been extensively reviewed by Waterman and Mole (1994), Schofield et al. (2001), Mueller-Harvey (2001), Hagerman (2002), Silanikove et al. (2001), and Mueller-Harvey (2006).

2.8.3 Colourimetric assays

The remainder of this section will focus upon chemical colourimetric methods, with emphasis on the acid-butanol assay. Total phenolics (including condensed tannins) are most frequently measured using either the Prussian Blue assay (Price and Butler 1977), the modified Prussian Blue assay (Graham 1992) or the Folin-Ciocalteu assay (Singleton and Rossi 1965). These methods are most appropriate when an unknown combination of plant species contributes a diverse spectrum of tannins and other phenols and polyphenols to a litter layer or an organic soil (Fierer et al. 2001; Kraus et al. 2003). Agronomic applications more commonly use the vanillin assay (Price et al. 1978; Hagerman 2002), which reacts with condensed tannins, as well as catechin monomers, or the more popular acid-butanol assay (Porter et al. 1986; Terrill et al. 1992; Wolfe et al. 2008), whose colour development is specific to condensed tannins (Hagerman 2002).

2.8.4 The acid-butanol assay

The current incarnation of the acid-butanol assay has evolved over a half-century from work initiated by Hillis and Swain (1959), through to modifications on the assay of Porter et al. (1986) by Terrill et al. (1992) and Wolfe et al. (2008). A detailed timeline of the method's early developments, the reader is directed to Waterman and Mole (1994). The assay depends upon acid-catalyzed oxidative depolymerisation of condensed tannins, which cleaves of inter-flavan bonds of the polyphenol to yield anthocyanidins (see Figure 2-5).

Anthocyanidins produce a bright pink to red pigmentation when heated in alcohol, and the resultant solution is measured spectrophotometrically against assayed standards of known condensed tannin concentration (Terrill et al. 1992). Quantitative interpretation of condensed tannin concentration is most accurate when samples are compared to

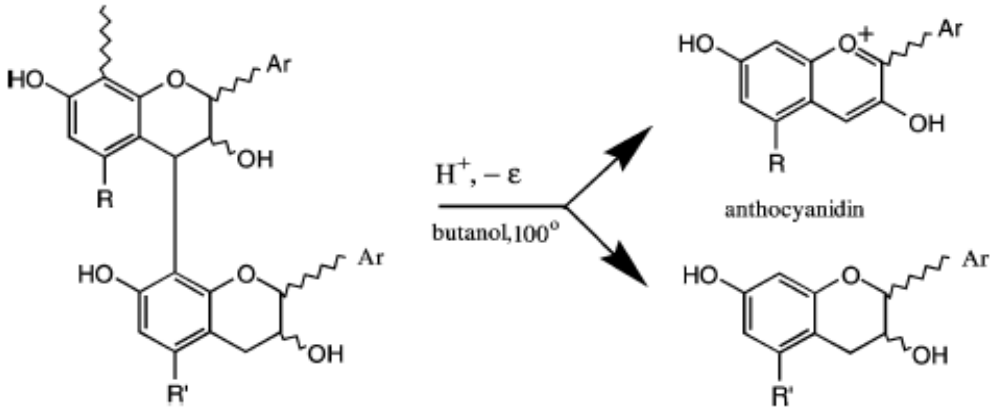


Figure 2-5: Chemistry of the acid-butanol reaction. Oxidative cleavage of the inter-flavan bond yields coloured anthocyanidin products.

standards of purified condensed tannins from like material (Mueller-Harvey 2006). Further analysis of protein and fibre-bound fractions are possible by assaying the products of an SDS/2-mercaptoethanol/Tris-Cl extraction, and the remaining residues, respectively (Terrill et al. 1992). Efforts to streamline this process via automatic continuous-flow methods have been unsuccessful (see Nitao et al. 2001).

Though widely-used in tanniniferous forage research, these methods are not without criticism (Waterman and Mole 1994; Makkar et al. 1999). They are subject to the technical precision of the researcher's hand at multiple junctures, are notoriously labour-intensive, and most importantly, their yield may not necessarily reflect true tannin activity in either soil or rumen environments. Colour development in assays is quantitative, and colour yield depends upon tannin conformation, inter-flavan linkages, and presence of 5-OH groups (Ferriera and Bekker 1996); these tannin structural differences have repeatedly been hypothesized to influence tannin activity in both soils and ruminants, thus, the value of colourimetric tannin assays depends upon their integration with tangible biological activity *in vivo*.

2.8.5 Tannins in manure – presence, extraction, and quantification

Throughout this review, condensed tannins have been shown to impact nutrient cycling in natural ecosystems, and nutrient availability when fed in forage-based diets to ruminant livestock. Despite recently improved understanding of condensed tannins on intake, ruminal digestion, gastrointestinal parasite burdens, and productivity, little is known about the fate of tannins in post-ruminal digesta, excreta, and their potential effects in manure. The pH-dependent hydrogen bonding, which is speculated to be the dominant attractive force between tannins and proteins, has largely been studied *in vitro* (Martin and Martin 1982). Quantification of post-ruminal dissociation, digestion, and absorption of tannin-bound proteins *in vivo* has been hindered by methodology which lacks the ability to detect conformational changes in condensed tannins and their presence in bound forms with digesta, feces, and manure. Availability of tannin-protein complexes for degradation distal to the acidic abomasum has been questioned (Terrill et al. 1994; Waghorn 2008). Furthermore, extraction and identification of tannins in digesta or feces has been unsuccessful using colourimetric assays (Terrill et al. 1994) and use of biological assays has not been fully explored (Gedir et al. 2005).

In light of these challenges to direct quantitative tannin measurement, researchers have taken an alternate approach by studying nutrient partitioning between urine and feces in tannin-containing diets (Powell et al. 2009; Grainger et al. 2009), the impact of this partitioning on ammonia emissions from barn floors (Misselbrook et al. 2005), and the subsequent effects on dairy slurry nutrient availability to crops (Powell and Grabber 2009). This shift towards higher fecal N excretion may indicate lower N absorption by a ruminant (de Klein and Eckard 2008), however, the organically-bound N in faeces is far less volatile than urea-N in urine. This organic sequestration of excreted N in feces would reduce or retard the process of denitrification, and subsequent emission of N_2O, a potent greenhouse gas, from livestock excreta. No quantitative attempts have been made

to connect these nitrogen dynamics in manure to tannin effects on biological activity further up the gastrointestinal tract of ruminants.

Powell et al. (2009) fed dairy cows silage-based diets of birdsfoot trefoil at two tannin levels (`low`` 0.56% and `high`` 1.66% DM diet), along with non-tannin containing alfalfa and red clover-based silages as controls. They assessed the effect of tannins on fecal and urinary nitrogen concentration and excretion, and found that the high-tannin trefoil diet significantly reduced urinary N excretion over red clover (7.1 g/L vs. 8.2 g/L), and the presence of tannin at either level increased fecal N excretion rates. The high tannin diet produced feces with significantly higher concentrations of NDF, nitrogen in both ADF and NDF, and relative amounts of fecal nitrogen compared to other silage types (Powell et al. 2009). A follow-up study by Powell and Grabber (2009) applied and incorporated the manure slurries from these respective diets on an N basis to silage corn. Though alfalfa, red clover, and two levels of tannin-containing birdsfoot trefoil produced slurries with different N, NH_4-N, and C:N ratios, their subsequent impacts on soil N, corn N, yield, and N recovery were not differentiable. Bioactivity and degradation of a heterogeneous organic amendment such as manure are difficult to predict without the added complexity of tannins. As illustrated by Powell and Grabber (2009), a more complete understanding of degradation and release of organically-bound nutrients will facilitate deeper research into tannin's influence in manure and soil.

2.8.6 Summary of tannin methods

Regardless of the method(s) employed to identify, quantify, or characterize condensed tannins, present research into tannin-containing forages is limited by a lack of an exhaustive predictive model for tannin activity based upon molecular structure, size, and charge. Until such a model is established, researchers are relegated to assembling a series of "partial pictures" of tannin concentration and characterization using a number of

analytical techniques, in order to make consistent assumptions of their activity *in vivo* (i.e. as facilitator for by-pass proteins in ruminant feedstuffs, as a non-pharmaceutical anthelmintic agent in sheep or goats, as carbon sequestration aid, etc.). More complete knowledge would vastly improve the dialogue between all relevant parties in integrated agricultural systems research, from molecular biologists to plant breeders, agronomists, ruminant nutritionists and pathologists, and most importantly, to producers.

2.9 Summary

Ecologically-sensitive and or agriculturally unsuitable lands have found refuge in policy-driven Conservation Reserve and Wetlands Reserve programs in the United States (USDA 2009), and producer-driven Alternative Land Use Services pilot programs in Canada (MAFRI 2010). These efforts recognize and value the duality of farmers and ranchers as both food producers and environmental stewards. By design, inclusion in these programs is limited to areas and ecosystems which may have escaped development and/or degradation at an earlier date had their non-agricultural environmental utility been appropriately valued.

Concurrently, substantially greater demands are being placed upon adjoining productive arable lands in order to meet the food requirements of an expanding population (Tilman et al. 2002), primarily in developing nations (de Haan et al. 1996). This strain is forecast to be further exacerbated by elevated demands for livestock products fed by external input-demanding grains (de Haan et al. 1996; Hendy et al. 1995). It is thus reasonable to assume that over time, the demand of environmental services, or non-production related ecosystem utility of presently arable lands will

similarly be taxed (Robertson and Swinton 2005). It is also reasonable to assume that a greater absolute demand for food, or that a greater production demand upon each presently available arable hectare will in turn create incentives for bringing marginal land into production if the process is left unregulated (Tilman et al. 2002). We would then be faced with the reality that our most productive lands must simultaneously also become our most well-conserved and functional ecosystems, and our food security will likely hinge upon their stewardship (Lal 2004).

The novel bioactive properties of forage-derived tannins may play an integral role in enhancing these ecosystem services within temperate arable grazing lands. As reviewed in the present study, they have shown to exert anthelmintic properties in grazing sheep (Niezen et al. 1995), thus reducing the frequency and dose required for parasite control of the limited repertoire of drugs. Sainfoin-derived polyphenols have also been shown *in vivo* to reduce generic *E. coli* shedding in cattle feces (Berard et al. 2009). Informed by studies of forest ecosystems, it has been theorized that the reactive nature of tannins in litter layers and organic soils may increase nitrogen synchrony, or decreasing the delay between susceptibility to loss (through leaching or mineralization) and plant uptake (Chapin III 1995; Clein and Schimel 1995; Bradley et al. 2000; Fierer et al. 2001). Robertson and Vitousek (2009) highlighted that similar phenomena in intact and diverse natural systems is far removed from the present reality of proliferous growth in short agricultural seasons. This present difference does not, however, interdict that tannin-producing forage species be selected for increased nitrogen use efficiency, such that future litter and soil nutrient dynamics support forages and crops with an increased ability to source both labile and organically-bound nitrogen forms. Lastly and most pressing is

the potential utility of tannins to significantly influence global carbon cycling, through two possible avenues: increased sequestration in soil organic carbon and non-humified organic litter (Nierop et al. 2006b), and reduction in greenhouse gas emissions from livestock production systems (Misselbrook et al. 2005; Powell et al. 2009; Bouchard, unpublished).

Each of the properties listed above have thus far been studied in relative isolation to one another. Cross-discipline references are notably absent in all but a handful of review papers, and inter-disciplinary study of site-specific systems involving tannins in either forest ecosystems or livestock production number one. To capture and exploit the bioactive properties of tannins in agricultural systems, there is a need for an integrated approach to quantifying the mediating processes in plant tannin production, tannin effects in ruminants, and subsequent tannin effects in both manure and soil. A basal understanding of each of these processes would better inform and be informed by improved and field-relevant laboratory analysis (Mueller-Harvey 2006). As explained by Robertson et al. (2004), such understanding is most valuable when achieved through systems-level research. The present study comprises a segment of such research, and as such, contributes to a proactive and anticipatory body of work investigating the potential roles of tannins in shaping our global environment.

3.0 RESEARCH HYPOTHESES AND OBJECTIVES

Hypotheses

Condensed tannins produced by sainfoin persist in a bioactive form in stockpiled manure after being fed to beef steers. Tannin bioactivity will decrease the short-term nutrient supplying ability of sainfoin manure over similar manure produced by alfalfa, a non tannin-containing legume. Altered nutrient bioavailability in concert with exogenous tannin application via sainfoin manure will influence tannin production in resident sainfoin plant material. These effects may further be positively affected if the tannin-producing legume is grown in a mixture with a non-tannin producing, competitive grass species rather than a monoculture.

Objectives

The primary objectives of the present study were:

1. To determine if tannin concentration in resident sainfoin plant material is altered by application of manure derived through feeding tannin-containing sainfoin hay forage to beef cattle.
2. To evaluate any difference in the nutrient bioavailability of alfalfa-derived and sainfoin-derived beef cattle manures, as measured through
    a. soil nutrient status,
    b. forage biomass accumulation and
    c. plant proximate analysis.
3. To determine if tannin concentration in sainfoin is altered by presence of meadow brome in a species mixture versus a pure sainfoin stand.

4.0 MATERIALS AND METHODS

4.1 Research site

The tannin experimental plots were located at the Ian N. Morrison University of Manitoba Research Farm in Carman, MB. (Latitude 49^{o} 30' N, longitude 98^{o} 02' W) on well-drained, fine sandy loam chernozemic soils (well-drained Hochfeld, sub-group Orthic Black of the Hibsin association). Growing season climate data for the location was obtained via the nearest weather station operated by the Manitoba Ag-Weather Program (Manitoba Agriculture, Food, and Rural Initiatives 2010), and these values were compared to 30 year long term averages from a nearby Environment Canada station (Environment Canada 2004). Weather data from May 1 to August 31was considered relevant to biomass accumulation, forage quality parameters, and plant tannin concentration of harvest dates in late June and late August of both years.

4.2 Experimental design

4.2.1 Forage plot layout and establishment

The plots consisted of five legume, grass, and legume-grass forage combinations:

1 Alfalfa (*Medicago sativa*)
2 Sainfoin (*Onobrychis viciifolia*)
3 Meadow brome (*Bromus biebersteinii*)
4 Alfalfa + Meadow brome
5 Sainfoin + Meadow brome

The five forage combinations were sown in the spring of 2006 on adjoining 4m x 8m plots in a randomized complete block design, separated by approximately 0.3m grass spacer rows. The seeding operation was performed using a Yanmar tractor pulling a no-till disc drill (Fabro Enterprises, Swift Current, SK) at a row spacing of 15cm and a shallow (<2cm) depth. Four replicates of each plot were sown approximately 4m apart, and were allowed one year to establish.

Species were selected for the trial on the basis of information available in the literature. Tannin-free *Medicago sativa* (Alfalfa) is the most common leguminous forage in the Northern Great Plains (Forster 1999). *Onobrychis viciifolia* (Sainfoin) is a common tannin-containing leguminous forage, often grown in pasture mixtures for both hay and grazing. It is well-represented in ruminant nutritional and pathogenic studies (Jones et al. 1994; Aerts et al. 1999; McMahon et al. 1999; Hoste et al. 2005; Scharenberg et al. 2007) as well as in agronomic or plant physiology work related to tannins (Lees 1986; Lees et al. 1993; Marais et al. 2000; Gebrehiwot et al. 2002).

Since plant investment in secondary metabolites varies considerably between location, year, species, and even cultivar, a preliminary screening trial was conducted in 2005 and 2006 for an assortment of tannin-containing fodder forages grown in western Canada, from which condensed tannin concentrations at both vegetative and mature growth stages were examined (Knox et al., unpublished). *O. viciifolia* was the second most proliferous tannin-producing species, behind the little-known perennial forb *Dalea purpurea*, or purple prairie clover, and was thus selected for the present study. *Bromus biebersteinii* (meadow brome) is a common forage grass, and was used as a

representative species in tame hay grass-legume mixtures with all species listed previously, as well as a tannin-free, non-leguminous control main plot forage treatment.

4.2.2 Manure source, analysis, and application

The manures supplied for this project were sourced from beef cattle feeding trials in Glenlea, Manitoba, where sainfoin represented the tannin-containing manure, and alfalfa represented the tannin-free manure (Bouchard, unpublished). The forages fed to the beef cattle were sourced from the Agriculture and Agri-Food Canada Research Centre in Brandon, Manitoba. Each of these forage species was fed in both silage and dry hay forms in the winter of 2006 in a feedlot scenario. The manure pack – including straw bedding – was stockpiled, and turned at bi-weekly intervals until the time of application to facilitate the composting process.

A representative composite sample of each stockpile was obtained from 10 sub-samples in late September 2007; these were each analyzed for pH, EC, moisture, carbon, nitrogen, sulphur, and total mineral content at Norwest Labs in Winnipeg, Manitoba. Results of this analysis were determined using the following methods: Nitrogen based on AOAC 993.13 – determination of total nitrogen in fertilizers (AOAC 2007), pH in a 1:5 slurry according to TMECC-04.11 (Thompson et al. 2002), electrical conductivity (EC) in a 1:5 slurry according to TMECC-04.10 (Thompson et al. 2002), and all minerals (phosphorus, potassium, magnesium, and sodium) by ICP-emission according to AOAC 985.01 (AOAC 2007).

The results of this analysis (Table 4-1 and Table 4-2) led to selection of sainfoin hay and alfalfa hay-derived manures as tannin and non tannin-containing manures,

respectively. This decision was made on the basis of their similar nutrient profiles and observed but unquantified physical similarities in particle size and stage of decomposition. A common mass-based rate of solid beef manure application of 44.8 t/ha (or 20 tons per acre) was chosen as the experimental rate in an effort to mimic a commercial tame pasture receiving stockpiled manure. Similar rates have been used in beef manure research elsewhere in Canada (Moolecki et al. 2004; Stumborg et al. 2007).

Manure nutrient content at this rate is presented in Table 4-3. Available nitrogen was calculated as per the MAFRI *Characterization of Solid Beef Manure* study reported by Loro (2005), whereby it is assumed that 25% of organic N is labile at application, and 25% of Ammonium N is volatilized. It was acknowledged that such an estimate of fall-applied and unincorporated solid beef manure may significantly over-estimate available N by not accounting for greater N volatilization losses, however, the undecomposed nature of the stockpiled manures created a conflict between the physical and chemical characteristics of their application. If a higher application rate was selected on the basis of higher expected N volatilization losses, the physical bulk of the manures – which contained substantial amounts of oat straw bedding, residual feedstuffs, along with cattle feces and urine – would have been an obvious physical impediment to spring regrowth in the year following manure application. Since hypotheses of the present study revolved around the effects of tannins in agricultural ecosystems and not the ability of forages to overcome adverse physical barriers to spring growth, a compromise was made between nutrient content and application rate.

Table 4-1: Nitrogen and sulphur content and physical properties of sainfoin and alfalfa manure on an as-applied wet-weight basis.

Manure Origin	Organic N (%)	Nitrate/Nitrite N (%)	Ammonium N (%)	Available N (%)	Total S (%)	Moisture (%)	EC (*dS/m*)	pH
Sainfoin Hay	0.82	0.0364	0.0542	0.282	0.180	68.5	69.5	9.4
Alfalfa Hay	0.79	0.0161	0.0406	0.244	0.190	68.2	71.4	9.9

Table 4-2: Mineral content of sainfoin and alfalfa manures on an as-applied, wet-weight basis

Manure Origin	Calcium	Phosphorous	P_2O_5	Potassium	K_20	Magnesium	Sodium
Sainfoin Hay	0.976	0.156	0.357	1.680	2.030	0.255	0.128
Alfalfa Hay	1.049	0.114	0.262	1.780	2.140	0.274	0.115

Table 4-3: Manure nutrient content (kg ha^{-1}) in sainfoin and alfalfa manures as applied to the forage plots at 44.8 t/ha rate.

Manure Origin	Applied Manure Nutrient and Mineral Content (kg ha^{-1})			
	Available N	Total S	P_2O_5	K_2O
Sainfoin Hay	126.4	80.6	159.9	909.4
Alfalfa Hay	109.3	85.1	117.4	958.7

To further lessen the possibility of a physical impediment caused by manure application, at three days prior to application the stockpiled manures were mechanically disturbed with a three-point hitch-mounted rotovator in order to break up large fragments. This tillage operation created a more consistent product, facilitating precise application, more uniform nutrient content, and easier handling.

The forage plots received an application of stockpiled solid beef cattle manure derived from both tannin (source: sainfoin hay) and non-tannin (source: alfalfa hay) containing forages at 44.8 t/ha on October 25, 2007. This is a common time and baseline rate for commercial application of stockpiled beef cattle manure. Manures were weighed in 80L bins (Rubbermaid), transported from Glenlea, MB. to Carman, MB., and then gently applied to the plots by shovel according to the prescribed treatment and rate. Each of the nine forage species and species mixture plots was split into thirds, receiving in randomized fashion one third sainfoin manure, one third alfalfa manure, and one third a manure-free control treatment. Experimental design, including factorial variables of plant species/species mixture and manure application was a randomized complete block design with a split plot arrangement (see Appendix 8-32 for plot plan).

4.3 Sample processing and data collection

4.3.1 Forage sampling and processing

Forage collection was partitioned into two harvest dates, or “cuts”, in both 2007 and 2008 (see Table 4-4 for summary of field operation dates). Harvest dates were staged to coincide with maturities of 75-100% bloom of the sainfoin and alfalfa, though it

was recognized that due to more advanced development of sainfoin prior to the first harvest, alfalfa would be slightly less mature than sainfoin at both harvest dates. In 2007, whole-plant samples were hand-harvested from a 1.0 m^2 area located in the approximate centre of each plot, bagged, then promptly returned to the laboratory and stored at -20^oC prior to processing. The process was utilized for the first and second harvest conducted in 2007, as well as the first harvest of 2008, where the sampling expanded to accommodate the split-plot manure and control treatments. In 2008, sampling size was reduced to 0.25 m^2 as this was deemed an adequate quantity of forage for yield estimation and subsequent proximate analysis

Frozen plant samples from both harvest dates in 2007 and the first harvest in 2008 were partially thawed immediately prior to sorting by species. The sorted biomass was then re-frozen while awaiting further processing. Material from the second harvest in 2008 was sorted by species under a shaded canopy in the field as fresh material, transported to the laboratory, and placed on room-temperature aeration drying beds.

In 2007, all forages destined for tannin analysis were sub-sampled from the primary whole-plant sample, and then treated separately. In 2008, a second sampling date following the primary harvest was reserved for tannin-containing species. In both sainfoin and sainfoin + meadow brome forage treatments, approximately 300-500g of whole sainfoin plants per plot were hand-harvested from a 1.0 m^2 area and then frozen prior to freeze-drying. This separate harvest minimized handling operations on tannin-containing plant material prior to tannin analysis.

Table 4-4: Manure application, plant sampling, and soil sampling timeline in 2007 and 2008 at Carman, MB.

Year	Field Operation		
	Plant Sampling	*Soil Sampling*	*Manure Application*
2007	1st Cut - June 27 2nd Cut - August 24	September 24-25	October 25
2008	1st Cut - June 26 1st Cut - June 27 (for tannin analysis) 2nd Cut - August 16 2nd Cut - August 19 (for tannin analysis)	October 12-13	-

Sorted biomass destined for proximate analysis was partially dehydrated on room-temperature aeration beds before being dried in a forced-air oven at 55^{o}C for 36-48 hours, and then weighed for dry matter yield. Sorted sainfoin samples destined for condensed tannin analysis were freeze dried in VirTis Genesis 25LE and VirTis 25LL freeze dryers (SP Industries, Gardiner, NY) as fresh, frozen samples, and then weighed for dry matter yield.

Both oven-dried and freeze-dried plant samples were ground using a Wiley Mill Model No.1 (Arthur H. Thomas Co., Philadelphia) to pass through a 1mm mesh screen. Ground plant samples were stored in sealed plastic bags prior to laboratory analysis. Ground samples destined for tannin analysis were stored in a freezer to mitigate the possibility of oxidative degradation prior to extraction and assay.

4.3.2 Soil nutrient analysis

Soil sampling for nutrient analysis occurred in the fall in both years at 0-15cm and 15-60cm depths. In 2008, the soil sampling protocol expanded to incorporate the sub-plot manure treatments. Using a Dutch auger with a five centimetre diameter bit, four subsamples at the 0-15 cm depth and two subsamples at the 15-60cm depth of each treatment in each replicate were composited. Samples were kept refrigerated until they were re-packaged and sent to commercial laboratories for analysis.

Analysis of soil samples was conducted at Bodycote Laboratories (Winnipeg, MB) in 2007 and at AgVise Laboratories (Northwood, ND) in 2008; the privatization of the Winnipeg Bodycote facility made sourcing another soil analysis provider necessary. Bodycote Laboratories methods were as follows: Nitrate-nitrogen (N) was determined by automated cadmium reduction, phosphorus (P) was determined by sodium bicarbonate extaction (Olsen method) and continuous flow colourimetry, sulphate-sulphur (S) by ICP, and potassium (K) by automated flame photometry method. Both pH and soluble salts (mhos/cm) were measured in a 1:2 soil:water suspension. Percent organic matter (OM) was determined by loss on ignition at 360^{o}C.

AgVise Laboratories methods were as follows: Nitrate-nitrogen (N) was determined by KCl extraction and cadmium reduction, phosphorus (P) was determined by sodium bicarbonate extaction (Olsen method), sulphate-sulphur (S) by KCl extraction and turbidimetric determination of barium sulphate precipitant, and potassium (K) by ammonium acetate extraction/ICP determination. Both pH and soluble salts (mhos/cm) were measured in a 1:1 soil:water suspension. Percent organic matter (OM) was determined by weight loss on ignition at 360^{o}C.

4.3.3 Plant proximate analysis

A suite of plant proximate analyses were performed on all forage samples, which measured ash (total mineral content), nitrogen (expressed as crude protein % DM), NDF, ADF, and mineral content, including Ca, P, K, Mg, and Na. Analysis of mixed species was conducted on the separate constituent components of each mixture (for instance, sainfoin and meadow brome). These separate species values were multiplied by percent dry matter species composition data to arrive at a mathematical whole-plot value.

Prior to nutrient and fibre analysis, analytical dry matter (DM) for each sample was determined (method 934.01; AOAC 1990). Oven-dried and ground plant samples were sub-sampled and analyzed for nitrogen (N) using a LECO CN-2000 (LECO, St. Joseph, MI). Crude protein content was calculated as 6.25 x N content. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) were analyzed in an Ankom 200 Fiber Analyzer (Ankom Technology Corporation, Fairport, NY). NDF was analyzed according to Van Soest et al. (1991) using α-amylase (Sigma No. A3306) and sodium sulphite (Anachemia No. 85514-380), and ADF was analyzed according to method 973.18 (AOAC 1990) adapted by Komarek et al. (1993). Alfalfa forage standards were included in each run of fibre analysis. Neutral detergent fibre content in sainfoin was not corrected for condensed tannin content.

Total ash in forages was based on AOAC Method 942.05 (1990), from which calcium (Ca), phosphorus (P), potassium (K), magnesium (Mg), and sodium (Na) were measured by inductively coupled plasma (IPC) emission spectroscopy (method 985.01; AOAC 2007) using a Varian Vista-MPX CCD Simultaneous ICP-OES (Varian Analytical Instruments, Walnut Creek, CA).

4.3.4 Plant tannin analysis

Condensed tannins present in sainfoin were extracted and quantified using a modified HCl-Butanol method outlined by Terrill et al. (1992) for "soluble" condensed tannins. A detailed description of the procedure is located in Appendix 8-33. Spectrophotometric readings were standardized against purified sainfoin condensed tannins obtained from a previous study in the laboratory of Dr. Yuxi Wang, Agriculture and Agri-Food Canada, Lethbridge Research Centre. The purified sainfoin standard material was prepared via a modification of Asquith and Butler (1985) by Hagerman (2002), and used in previously published tannin studies in western Canada (Wang et al 2007). An average of three standard curves was used to correlate spectrophotometric values with quantitative tannin concentration, and a common sainfoin material was inserted in duplicate in each extraction and assay of plant material to ensure homogeneity of the procedure over time. Whole plot condensed tannin concentration in the sainfoin + meadow brome mixtures were calculated by multiplying sainfoin tannin values by the percentage of sainfoin dry matter in the mixture. This mathematical assumption of whole plot tannin concentration was validated by laboratory assays in which meadow brome was consistently not differentiable from non-tannin containing blanks in HCl-Butanol colourimetric assays.

4.4 Statistical analysis

4.4.1 Means comparisons and treatment estimates

Comparisons on whole-plant sainfoin condensed tannin concentration were made were between years, manures, and harvest dates separately. Interactions of

manure*harvest date, year*harvest date, or forage stand*year are biologically inexplicable given the current state of knowledge on tannin dynamics utilizing current methodology.

Year, main-plot forage treatment, and sub-plot manure treatment effects were all tested using analysis of variance (ANOVA) for all measurements. ANOVA was performed using Proc Mixed (SAS Institute Inc. 2004) on plant proximate analysis, forage biomass yield, and sainfoin tannin concentration variables separately for each harvest date while soil nutrient analysis was performed over the entire season. The underlying assumptions of normality within ANOVA were tested using Proc Univariate, where the Shapiro-Wilk test for normality was observed along with schematic box plots. Visual diagnostics of homogenous residual distribution was observed through Proc Plot. Estimates between treatment effects of the two manures were used in all plant measurements in 2008. Means comparisons were considered significantly different at $P < 0.05$, and trends were considered statistically relevant at $P < 0.10$.

4.4.2 Stepwise regression

Multiple linear regression was applied to both soil nutrient analysis and plant proximate analysis using the stepwise modification of the forward selection technique, with plant tannin concentration as the independent variable (SAS Institute Inc. 2004). The default alpha level of 0.15 was used for dependent variable selection. The stepwise regression procedures were not expected to provide predictive or quantitative modeling parameters, rather, they were used to better disseminate the analyzed soil and plant

parameters associated with sainfoin tannin concentration. Similar sampling schedules between years and harvest dates facilitated inclusion of all data in stepwise models.

5.0 RESULTS AND DISCUSSION

Monthly weather data from the Carman Manitoba Ag-Weather Station is summarized along with long-term average monthly data from the nearby Graysville, MB. Environment Canada station (Environment Canada 2004) in Table 5-1.

Table 5-1: Mean monthly air temperature and precipitation during the growing season (MAFRI 2010) and long term climate averages (Environment Canada 2004) at Carman, MB.

Year	May	June	July	August	Growing Season [2]
Air Temperature (^{o} C)					
2007	12.3	17.4	20.9	17.3	17.0
2008	8.9	15.3	18.0	19.1	15.3
Long-Term Average [1]	12.4	17.2	19.7	18.1	16.9
Precipitation (mm)					
2007	107.8	81.8	58.0	23.6	271.2
2008	33.6	84.4	37.6	54.8	210.4
Long-Term Average [1]	59.8	75.5	73.5	66.8	275.6

[1] 30 year average

[2] Growing season defined as May 1 - August 31

5.1 Forage production

Dry matter biomass yields in the non-manured control treatment increased from 2007 to 2008 (Table 5-2). The first cut biomass was significantly greater ($P < 0.05$) than the second cut in 2008 for both meadow brome and alfalfa + meadow brome forages, and this trend ($P < 0.10$) was also observed in the sainfoin monoculture. Second cut biomass

yields did not follow this pattern, as only the sainfoin + meadow brome forage showed a significant difference ($P < 0.01$) between years.

Table 5-2: Comparison 2007 vs. 2008 dry matter biomass (kg/ha) of the five forages in the non-manured control treatment.

Cut	Year	Forage Stand Dry Matter Biomass (kg ha $^{-1}$)				
		Alfalfa	*Meadow Brome*	*Sainfoin*	*Alfalfa + Meadow Brome*	*Sainfoin + Meadow Brome*
Cut 1	2007	3200	3346 b	3519	3688 b	3828
	2008	4538	5306 a	5605	5260 a	5664
	P > F	0.2447	0.0185	0.0675	0.0141	0.1632
	SE	509	305	575	380	635
Cut 2	2007	2443	1057	1278	3658	1563 b
	2008	3317	1210	1635	3272	2852 a
	P > F	0.2742	0.3970	0.5412	0.2344	0.0026
	SE	550	124	387	307	251

Fall application of commercial rates of solid beef cattle manure did not affect forage DM biomass yield in the subsequent growing season (Table 5-3). The sole exception to this was observed in the second cut of the sainfoin + meadow brome mixture, where sainfoin-derived manure application resulted in a significant ($p < 0.05$) reduction in forage biomass over the non-manured control. A similar trend ($P < 0.10$) was observed in the first cut of the sainfoin monoculture, where application of sainfoin manure increased dry matter biomass by 1009 kg/ha over the non-manured control, and 1163 kg/ha over the alfalfa manure treatment (estimate between manures was significantly different at $P < 0.05$).

Manure origin was insignificant in nine out of ten biomass comparisons in 2008 (Table 5-3). The first cut of sainfoin monoculture in 2008 showed that sainfoin-derived manure application yielded 1163 kg/ha DM biomass greater than that grown with alfalfa-

derived manure, however, the difference between sainfoin manure and the control was insignificant. Application of either manure did not significantly influence DM species

Table 5-3: Dry matter biomass (kg/ha) of the five forages under three manure sub-plot treatments in 2008.

Cut	Manure Treatment	Forage Stand Dry Matter Biomass (kg ha $^{-1}$)				
		Alfalfa	*Meadow Brome*	*Sainfoin*	*Alfalfa + Meadow Brome*	*Sainfoin + Meadow Brome*
Cut 1	Alfalfa Manure	4504	6296	5451 b	4873	6638
	Control (no manure)	4538	5306	5605 b	5260	5664
	Sainfoin Manure	5571	5757	6614 a	5443	6499
	P > F	*0.1777*	*0.4702*	*0.0597*	*0.5093*	*0.5202*
	SE	*413*	*727*	*541*	*382*	*769*
	P>F A-S Estimate	*ns*	*ns*	*0.0306*	*ns*	*ns*
Cut 2	Alfalfa Manure	2360	1452	1693	3164	2342 ab
	Control (no manure)	3317	1210	1635	3272	2852 a
	Sainfoin Manure	3817	1467	2297	2727	1807 b
	P > F	*0.4044*	*0.6183*	*0.0923*	*0.6330*	*0.0202*
	SE	*623*	*216*	*316*	*505*	*280*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*0.0861*

composition within mixed-species plots in 2008 (see Appendix 8.1 to 8.3), nor did it significantly alter forage production of the five main plot treatments (alfalfa, sainfoin, meadow brome, alfalfa + meadow brome and sainfoin + meadow brome) relative to one another (Table 5-4).

Comparisons between annual DM biomass yields in the present study are within the range of published annual values for alfalfa (Hanna et al. 1977), sainfoin (Goplen et al. 1991; Waddington et al. 1986; Cooper 1972), and meadow brome (Knowles et al. 1993), though the two-cut partitioning of annual biomass production has not been explicitly studied. Specific yield values for dryland alfalfa + meadow brome and sainfoin + meadow brome mixtures in the northern Great Plains are not available for comparison.

Neither the weak fertility effects of the two manures used in this experiment, nor the cooler temperatures and more uniform precipitation distribution of the 2008 growing season (Table 5-1) were enough to positively impact forage productivity. It was anticipated that even if the nitrogen availability of sold beef cattle manure was less than

Table 5-4: Comparison of the five main-plot forage dry matter biomass yields (kg/ha) in the control year in 2007, and under three manure sub-plot treatments in 2008. Estimates between forages alfalfa and sainfoin, and alfalfa+ brome and sainfoin+brome are included.

Cut	Forage	Forage Biomass (kg ha^{-1}) by Year and Manure Treatment			
		2007 *Control*	2008 *Alfalfa Manure*	2008 *Control*	2008 *Sainfoin Manure*
Cut 1	Alfalfa	3199	4504	4538	5571
	Alfalfa + Meadow Brome	3687	4874 b	5260	5444
	Meadow Brome	3346	6296	5306	5757
	Sainfoin	3518	5451	5604	6614
	Sainfoin + Meadow Brome	3827	6638 a	5664	6499
	P > F	*0.7754*	*0.0643*	*0.6473*	*0.3761*
	SE	*375*	*610*	*593*	*561*
	P>F A-S Estimate	*0.5636*	*0.2286*	*0.2051*	*0.1688*
	P>F AMix-SMix Estimate	*0.7984*	*0.0357*	*0.6209*	*0.1640*
Cut 2	Alfalfa	2443 a	2360	3317 a	3817 a
	Alfalfa + Meadow Brome	2658 a	3164	3272 a	2727 ab
	Meadow Brome	1057 b	1452	1209 c	1467 c
	Sainfoin	1277 b	1693	1635 bc	2297 bc
	Sainfoin + Meadow Brome	1563 b	2342	2852 ab	1807 bc
	P > F	*0.0012*	*0.0869*	*0.0092*	*0.0207*
	SE	*290*	*404*	*407*	*438*
	P>F A-S Estimate	*0.0044*	*0.2757*	*0.0139*	*0.0302*
	P>F AMix-SMix Estimate	*0.0064*	*0.1841*	*0.4857*	*0.1627*

the values which informed the manure treatment application rate (Loro 2005), some nitrogen fertility effect of the manure would be realized in dry matter forage yields. Conversely, manure application occasionally had deleterious but statistically insignificant effects on yield, and these effects were more noticeable when alfalfa manure was applied. The typical explanation for depressed production in the year following solid cattle manure application is net N immobilization due to the inherently high C:N ratio of

manure containing bedding material and residual feedstuffs (Quian and Schoenau 2002), however, soil test values of the present study do not support this hypothesis.

The sum of these observations suggests that the mid-October application of solid beef cattle manure may have affected plant health and thus productivity in the following growing season. Since additional measures were taken to mitigate the possibility of direct physical damage by solid manure application, such as rotary tillage of stockpiled manure to ensure small and uniform particle size prior to application, and hand application of manure to the forage plots, it was unlikely that physical damage occurred to the forages above that seen in commercial production scenarios. It is thus likely that the volume of solid manure applied in the late fall, when the forages were in a near-dormant growth state, may have been a physical impediment to re-growth in the subsequent season.

The results of the present study suggest that the long-term fertility and soil organic matter effects of manure amendment to hayed forages are not apparent in dry matter biomass production within the one-year timeframe of this experiment.

5.2 Soil nutrient status

5.2.1 Year effect on a non-manured control treatment

Fall soil test nutrient differences between 2007 and 2008 non-manured control treatments varied between the five main plot forage treatments (Table 5-5).

Measurement of fall soil test nitrogen showed that in the surface (0-15 cm) soils, only alfalfa + meadow brome forage reduced soil nitrogen from 2007 (41 kg/ha) to 2008

Table 5-5: Fall soil test measurements at 0-15, 15-60, and 0-60 cm depths in 2007 and 2008 under non-manured control treatments.

Forage Stand	Year	0-15 cm							15-60 cm		0-60 cm	
		N (kg/ha)	*P (ppm)*	*K (ppm)*	*S (kg/ha)*	*pH*	*Salts (mmhos/cm)*	*OM (%)*	*N (kg/ha)*	*S (kg/ha)*	*N (kg/ha)*	*S (kg/ha)*
Alfalfa	**2007**	35	10	137	12	6	0.25	4.0	52	40	87	52
	2008	34	5	122	13	5	0.22	3.9	51	123	76	122
	P > F	0.6689	0.0105	0.4094	0.6042	0.0774	0.1328	0.3189	0.8077	0.2972	0.7745	0.2763
	SE	2.7376	0.6770	26.8991	1.4068	0.1524	0.0121	0.3812	3.3541	53.1168	4.6402	52.6765
Alfalfa + Meadow Brome	**2007**	41	11	111	13	6.0	0.28	3.9	62	197	103	209
	2008	24	6	154	17	5.8	0.21	4.2	35	175	53	171
	P > F	0.0414	0.0590	0.2817	0.2251	0.0689	0.1847	0.0141	0.0781	0.8041	0.0330	0.8382
	SE	4.2032	1.1180	27.3816	2.8994	0.2234	0.0309	0.2481	9.9158	106.4800	12.1942	109.2000
Meadow Brome	**2007**	19	10	120	10	5.8	0.19	4.0	30	72	49	82
	2008	8	7	128	14	5.5	0.14	3.9	11	47	17	55
	P > F	0.0950	0.1098	0.3704	0.0796	0.1177	0.1871	0.0154	0.0290	0.5860	0.0068	0.6394
	SE	2.7890	1.6520	23.4902	1.1319	0.1203	0.0144	0.2991	3.1058	32.5581	2.8922	32.7487
Sainfoin	**2007**	25	10	105	10	5.9	0.22	3.9	41	66	66	77
	2008	27	6	120	10	5.7	0.19	3.8	25	64	46	66
	P > F	0.7688	0.0228	0.3538	0.8543	0.0032	0.1027	0.1170	0.0381	0.8463	0.1892	0.8343
	SE	3.8161	0.6124	19.2484	1.0945	0.0559	0.0115	0.2737	2.5900	17.6018	6.2367	17.2648
Sainfoin + Meadow Brome	**2007**	27	9	111	10	5.8	0.23	4.0	38	96	65	106
	2008	17	7	128	15	5.6	0.14	4.0	13	91	27	95
	P > F	0.1188	0.0486	0.0427	0.0120	0.1612	0.0160	0.8820	0.0038	0.9235	0.0146	0.9964
	SE	2.5475	0.6997	16.9555	1.3769	0.0989	0.0184	0.4107	3.1606	34.6282	4.0863	35.6644

(28). The effect of year had a greater impact on the 15-60 cm soil test nitrogen levels, with significant ($P < 0.05$) reductions in meadow brome, sainfoin + meadow brome, and sainfoin. The trend continued in alfalfa + meadow brome ($P = 0.0781$), but not in alfalfa. The combination of lower nitrogen levels at both depths led to significant 0-60 cm decreases in soil test nitrogen in 2008 compared to 2007 in alfalfa + meadow brome, meadow brome, and sainfoin + meadow brome. From these results, we can conclude that addition of meadow brome to a leguminous forage stand significantly decreased soil test nitrogen in the 0-60 cm depth when compared to the associated legume monoculture.

Phosphorus in the 0-15 cm soils significantly declined in alfalfa, sainfoin + meadow brome, and sainfoin monoculture treatments. This difference was not consistent across plant type or species. All other soil test measurements by year were within expected values, and any change in values between the years was not consistent between forages.

Since the forage plots received no exogenous fertility sources since their establishment in 2006, and all forage biomass was removed from the site via mechanical harvest, it was expected that residual nitrogen, phosphorus, and sulphur levels would decline over time in both soil test depths. Nitrogen fixation by sainfoin and alfalfa may offset or eliminate this decline in residual soil test nitrogen, however, in the legume-grass mixture plots, legume nitrogen fixation as seen in residual nitrate-nitrogen soil test values was susceptible to uptake by meadow brome. This potential for inter-crop cycling of nitrogen may not have been detectable by a single soil sampling date per year.

5.2.2 Solid beef cattle manure effects on 2008 soil test measurements

Application of 44.8 t/ha of either sainfoin or alfalfa-derived solid beef cattle manure in the fall of 2007 did not significantly alter soil nutrient status in the fall of 2008 (Table 5-6). The only soil nutrient parameter showing significantly different values from the non-manured control treatment was potassium (K) in the 0-15cm soil layer, in which application of either manure type increased K ppm by 137-308% across four of the five forage types. In only the sainfoin + meadow brome forage did the sainfoin-derived manure create a significantly ($P < 0.05$) greater estimate of soil potassium concentration from the alfalfa-derived manure; the remainder of significant manure effects did not differ by manure origin. Previous studies on solid cattle manure have found that after repeated annual or biannual applications, K will accumulate as the dominant exchangeable cation in surface soil layers (Hao and Chang 2002; Moolecki et al. 2004), however, evidence of substantial changes within one growing season have not been documented.

This study did not demonstrate a relationship between fall application of solid beef cattle manure and changes in soil nutrient status in the subsequent year, nor was there evidence to suggest that the nutrient availability of beef cattle manure to perennial forages differed between manure origins. It was hypothesized that sainfoin-derived manure potentially possessed distinct "tannin-like" properties analogous to those observed in ruminant nutrition studies (Waghorn et al. 1987), where nitrogen dynamics are temporally (Waghorn 1994) and spatially (Wang et al. 2007) altered through reversible tannin-protein complexes (Hagerman and Butler 1980). Conservation of these tannin properties in excreta and subsequently in stockpiled manure would factor into

Table 5-6: Means comparison of 2008 fall soil test measurements between three manure treatments. Estimates between alfalfa-derived and sainfoin-derived manures are included for each forage stand.

Forage Stand	Manure Treatment	0-15 cm							15-60 cm		0-60 cm	
		N (kg/ha)	*P (ppm)*	*K (ppm)*	*S (kg/ha)*	*pH*	*Salts (mmhos/cm)*	*OM (%)*	*N (kg/ha)*	*S (kg/ha)*	*N (kg/ha)*	*S (kg/ha)*
Alfalfa	Alfalfa	69	6	291 a	23	5.4	0.30	4.0	119	89	168	100
	none	34	5	122 b	13	5.3	0.22	3.9	51	123	76	122
	Sainfoin	48	6	215 b	23	5.4	0.27	4.0	64	55	100	70
	P > F	0.2552	0.2160	0.0357	0.3092	0.1100	0.2993	0.3989	0.2002	0.5130	0.2118	0.5684
	SE	13.9809	0.7071	34.7858	3.9493	0.0755	0.0304	0.3792	26.9499	44.9396	40.0387	44.2495
	P > F A-S Est.	ns	ns	0.1683	ns	ns	ns	ns	ns	ns	ns	ns
Alfalfa + Meadow Brome	Alfalfa	26	6	205	19	5.8	0.23	4.0	33	215	53	119
	none	24	6	154	17	5.8	0.21	4.2	35	175	53	171
	Sainfoin	52	8	211	17	5.7	0.28	4.0	42	138	84	138
	P > F	0.0901	0.2989	0.6188	0.8696	0.6787	0.1920	0.1072	0.4563	0.4144	0.1297	0.3688
	SE	8.8612	1.0897	45.5930	2.6693	0.1945	0.0379	0.2176	7.2222	83.1013	15.2643	82.6348
	P > F A-S Est.	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Meadow Brome	Alfalfa	15	8	395 a	24	5.8	0.21	4.2	15	133	27	140
	none	8	7	128 b	14	5.5	0.14	3.9	11	47	17	55
	Sainfoin	19	8	310 a	19	5.8	0.20	4.0	18	136	33	49
	P > F	0.2594	0.5596	0.0073	0.0755	0.0578	0.0790	0.1938	0.0963	0.4245	0.1242	0.3742
	SE	4.5871	1.3514	44.7821	2.4594	0.1090	0.0257	0.2914	1.9703	57.9388	6.0116	57.3654
	P > F A-S Est.	ns	ns	0.1704	ns	ns	ns	ns	ns	ns	ns	ns
Sainfoin	Alfalfa	43	7	246 a	18 a	5.6	0.25	3.9	30	151	65	61
	none	27	6	120 b	10 b	5.7	0.19	3.8	25	64	46	66
	Sainfoin	33	9	239 a	16 a	5.8	0.23	4.0	29	176	55	172
	P > F	0.1019	0.0610	0.0021	0.0211	0.3527	0.0641	0.3448	0.3971	0.2410	0.1299	0.2123
	SE	3.7722	0.6972	20.4315	1.3819	0.0846	0.0195	0.2842	2.6167	59.2950	5.8678	58.9253
	P > F A-S Est.	ns	ns	0.7620	0.4264	ns	ns	ns	ns	ns	ns	ns
Sainfoin + Meadow Brome	Alfalfa	17	8	337 a	19	6.2	0.24 a	4.0	15	81	28	89
	none	17	7	128 c	15	5.6	0.14 c	4.0	13	91	27	95
	Sainfoin	15	8	249 b	18	5.9	0.17 b	4.0	13	79	25	87
	P > F	0.8565	0.1985	0.0007	0.2306	0.0505	0.0001	0.8240	0.2633	0.9396	0.7162	0.9694
	SE	3.7657	0.7169	23.3427	1.2332	0.1588	0.0158	0.4002	1.4313	28.7700	4.1758	29.5704
	P > F A-S Est.	ns	ns	0.0163	ns	ns	0.0005	ns	ns	ns	ns	ns

degradation rates of bound organic matter, lability of soluble nutrients, and finally, in nutrient supply to the soil resident plants.

Three possible, but uninvestigated, scenarios may explain the lack of significance between sainfoin and alfalfa-derived manures:

1. Re-complexation of hydrolyzed tannin monomers into oligomeric and polymeric compounds which retain the defined structures, bioactivity, and solubility of condensed tannins (Haslam 1996) distal to the acidic abomasum does not occur. The resulting excreted tannin products thus will not influence nutrient availability or organic matter degradation beyond their constituent chemical value.
2. Assuming intact and/or conformationally-altered condensed tannins exist in excreted feces, non-reversible complexation with other macromolecules, minerals, and organic material (Terrill et al. 1994) over time minimizes their effects upon soil and plant-available nutrients. These condensed tannins themselves may also be susceptible at any stage to metabolism or abiotic degradation (Palm and Sanchez 1991; Kalburtji et al. 1999), which may additionally mitigate their bioactivity as measured in soil test values.
3. The soil fertility effects of solid beef cattle manure, irrespective of plant species origin, are not realized within the time scale of this experiment. In this scenario, the additional effects of tannins upon organic matter degradation and nutrient availability may only be realized in later seasons. It was anticipated that due to its polyphenolic content, nutrient bioavailability of sainfoin-derived beef manure would be less than that of a non-tannin containing alfalfa-derived manure, and that this difference would be captured in fall soil nutrient values. Apart from

potassium in the 0-15 cm soil layer, the alfalfa manure did not significantly alter soil nutrient content compared to a manure-free control treatment in 2008. Consequently, depressed nutrient availability in sainfoin manure could theoretically not be verified unless soil nutrient values fell below those of the manure-free control.

Current tannin methodology precludes analysis of the first two scenarios, though their study has strong potential impacts on greater systems-level nutrient cycling. Given that the alfalfa-derived manure produced equally variable and unpredictable results on soil fertility across repetitions and forage treatments as the hypothetically tannin-containing sainfoin-derived manure, the third theory appears most probable.

Four interrelated factors may explain the low and variable nutrient availability in the two manures, two which are related to the manure itself, and two related to the experimental design:

1. Solid manure as an organic amendment is extremely heterogeneous, even within a given site and feed source (Davis et al. 2002). In spite of efforts to mix and manipulate the stockpiles prior to manure application, the experimental design and sampling size may have not been large enough to capture differences within each stockpile.
2. Application of solid beef manure often contains low levels of ammonium N (Davis et al. 2002) and a carbon to nitrogen (C:N) ratio greater than 15:1, a value beyond which short-term N availability to the soil is limited (Qian and Schoenau 2002). It has been shown that when cattle diets are formulated to contain similar protein and energy content, grain:forage content does not

affect the chemical composition of the resultant manure pack (Boadi et al. 2004). The present study applied manure that was derived from forage diets.

Previous investigations of soil manure nutrient availability and accumulation (Beauchamp 1983; Mooleki et al. 1994; Chang and Janzen 1996; Stumborg et al. 2007) have used conventional, grain and silage-based feedlot manures. Typical feedlot diets are formulated for liveweight gains, are not standardized against a forage equivalent for energy or protein content, and thus contain higher concentrate levels such as those seen in Beauchemin and McGinn (2005). It is reasonable to assume that their resultant manure pack of energy and protein-dense diets would contain higher levels of nitrogen and lower levels of fibre, and thus a lower, more available C:N ratio. Manures of the present study may thus exhibit lower nutrient availability compared to those used in previous Canadian beef manure research.

3. Fall broadcast and unincorporated manures are susceptible to NH_3-nitrogen volatilization (Sutton 1994; Sanderson and Jones 1997). This factor may have been exacerbated by the 6-7 month lag between application and potential crop uptake, where the balance of that period was characterized by frozen soils and little biological activity.
4. Based upon the principles in points 2 and 3, the 44.8 t/ha application rate may have contained insufficient available nutrients to cause a significant change in soil nutrient status over one season. Other beef manure research has often used significantly greater mass-based application rates of manure to gauge nutrient availability to crops (Moolecki et al. 2004; Olson and Papworth 2006;

Miller et al. 2009). Comparable rates in a study on incorporated beef manure in annual cropping did not detect any changes in residual soil nitrate-nitrogen after five years (Stumborg et al. 2007), however, quantitative mass-based comparisons between studies are notoriously unreliable given the heterogeneity of solid beef manure.

The relevance of time scale to the study of tannin effects *in vivo* is highlighted by analogous studies on fertility and ecological succession in forest research. Almost without exception, the organic litter and soil layers which exhibit strong tannin-related influence on nutrient dynamics (see Schimel et al. 1996; Kraus et al. 2004b; Nierop et al. 2006; Kraal et al. 2009; Wurzburger and Hendrick 2009) likely accumulated over tens to hundreds of years. Agricultural systems will not likely require centuries to respond to tannin amendment; however, it is presently presumptuous to assume that CT will behave as efficiently and predictably in field studies as do inorganic fertilizers. Until tannin quantification and characterization methods can accurately be modelled to predict *in vivo* bioactivity – for instance, to establish a known "tannin response curve" - research will be limited to indirect observation of tannin cycling and subsequent ecosystem effects on nutrient cycling.

5.3 Plant proximate analysis

Proximate analysis values observed in the current study (represented in Tables 5-7 through 5-9) are within previously published ranges for sainfoin (Carleton et al. 1968; Koch et al. 1972; Kaldy et al. 1979; McGraw and Marten 1986; McMahon et al. 1999;

Frame 2005; Iwaasa et al. 2006), alfalfa (Parker and Moss 1981; Kidambi et al. 1990; Beauchemin and Iwaasa 1993; McMahon et al. 1999; Cassida et al. 2000; Iwaasa et al. 2006), meadow brome (Knowles et al. 1993; McCaughey and Simons 1999; Ferdinandez and Coulman 2001), and alfalfa + brome (Spandl and Hesterman 1997). Proximate analysis for the sainfoin + meadow brome mixture has not been studied previously, and no published comparisons are available.

Within all five main plot forage treatments there were frequent yet inconsistent significant differences between the two manure treatments and their associated non-manured control. Calcium and magnesium concentrations were generally lower under the two manure treatments, while potassium concentration was higher. Though small and inconsistent, these differences show more substantial evidence of a manure fertility effect that was not captured in residual soil test values measured after the end of the 2008 growing season, though variability between repetitions and harvest dates inhibits any consistently significant statistical conclusion on the fertility effect of either of the manures upon forage quality.

Comparisons within each forage species grown in monoculture versus a legume-grass mixture illustrate that proximate analysis of alfalfa and sainfoin does not significantly change with stand type (monoculture vs. mixture). Meadow brome, on the other hand, showed consistently significant higher first cut NDF and ADF values in sainfoin mixtures than when grown with alfalfa. This difference was likely a function of the increased competitive ability of alfalfa on meadow brome growth versus sainfoin, though maturity was not objectively quantified in the experiment. Details on competitive abilities of the two legume species in mixtures with meadow brome are

Table 5-7: 2008 plant proximate analysis of five forages in the non-manured, control treatment at the two harvest dates. All parameters are expressed on a % dry matter basis. Species mixture proximate values were analyzed by species, then mathematically combined on a % dry matter species composition basis.

Cut	Forage	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	Alfalfa	9.4178 a	20.0298 a	42.1092 c	34.0180 c	1.9403 a	0.1873 b	2.0229 a	0.4045 a	0.3886
	Alfalfa + Meadow Brome	9.1177 a	15.4034 b	47.7511 c	33.8422 c	1.4470 b	0.1716 bc	2.1842 a	0.3848 a	0.3855
	Meadow Brome	6.2834 b	5.8334 d	70.3647 a	42.8284 a	0.2653 c	0.1458 c	2.0884 a	0.1013 c	0.3498
	Sainfoin	6.1673 b	13.8732 b	43.9803 c	38.3258 b	1.0603 b	0.2392 a	1.4386 b	0.3493 a	0.3573
	Sainfoin + Meadow Brome	6.0056 b	9.2488 c	61.8580 b	41.7388 a	0.4877 c	0.1698 bc	1.9739 a	0.1971 b	0.3807
	P > F	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*0.0036*	*0.0124*	*<0.0001*	*0.2636*
	SE	*0.2864*	*1.9046*	*1.9407*	*1.1013*	*0.1378*	*0.0119*	*0.1341*	*0.0259*	*0.0159*
	P>F A-S Estimate	*<0.0001*	*<0.0001*	*0.4938*	*0.0095*	*0.0007*	*0.0162*	*0.0078*	*0.1764*	*0.1496*
	P>F AMix-SMix Estimate	*<0.0001*	*<0.0001*	*0.0002*	*0.0001*	*0.0003*	*0.8806*	*0.2729*	*0.0004*	*0.8173*
Cut 2	Alfalfa	8.4038 b	19.9733 a	38.8886 cd	31.6109	1.6038 a	0.1841	2.0345 a	0.3285	0.4177
	Alfalfa + Meadow Brome	8.0060 bc	17.8852 ab	44.6717 bc	33.4745	1.2669 a	0.1712	2.0962	0.3482	0.4139
	Meadow Brome	10.2968 a	9.8487 d	59.1461 a	37.8049	0.4897 c	0.1775	1.9199	0.2398	0.3901
	Sainfoin	6.8136 d	16.2958 ab	34.9249 d	31.2725	1.3200 a	0.1833	1.4067 b	0.4180	0.3734
	Sainfoin + Meadow Brome	7.1160 cd	14.6253 c	45.7810 b	35.6157	0.8960 b	0.1806	1.8563	0.3361	0.3691
	P > F	*<0.0001*	*<0.0001*	*<0.0001*	*0.0723*	*0.0002*	*0.9108*	*0.0995*	*0.3225*	*0.3661*
	SE	*0.3350*	*2.2278*	*2.0450*	*1.6753*	*0.1323*	*0.0100*	*0.1759*	*0.0567*	*0.0213*
	P>F A-S Estimate	*0.0051*	*0.0025*	*0.2172*	*0.8864*	*0.1088*	*0.9588*	*0.0243*	*0.2776*	*0.1538*
	P>F AMix-SMix Estimate	*0.0796*	*0.0054*	*0.7218*	*0.3741*	*0.0428*	*0.5450*	*0.3446*	*0.8811*	*0.1502*

Table 5-8: 2008 plant proximate analysis of five forages in the alfalfa manure treatment at the two harvest dates. All parameters are expressed on a % dry matter basis. Species mixture proximate values were analyzed by species, then mathematically combined on a % dry matter species composition basis.

Cut	Forage	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	Alfalfa	9.4587 a	17.6477 a	48.9021 d	35.9677 bc	1.5087 a	0.1902 b	2.8018 a	0.3176 a	0.4053
	Alfalfa + Meadow Brome	8.7032 a	14.3893 b	51.3356 c	35.5254 c	1.1930 ab	0.1754 c	2.4271 b	0.3231 a	0.3967
	Meadow Brome	6.9638 b	6.7498 d	71.9410 a	43.9007 a	0.2683 c	0.1264 c	2.3991 b	0.1018 c	0.3420
	Sainfoin	6.6265 b	13.5214 b	43.2876 d	38.4334 b	0.9354 b	0.2440 a	1.8389 c	0.3233 a	0.3632
	Sainfoin + Meadow Brome	6.5961 b	9.0872 c	61.9645 b	41.7218 a	0.4684 c	0.1887 b	2.3431 b	0.1686 b	0.4186
	P > F	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*<0.0001*	*0.0003*	*<0.0001*	*<0.0001*	*0.8080*
	SE	*0.3074*	*2.0442*	*1.9862*	*0.8876*	*0.1166*	*0.0119*	*0.1314*	*0.0186*	*0.0487*
	P>F A-S Estimate	*<0.0001*	*0.0012*	*0.8430*	*0.0788*	*0.0052*	*0.0081*	*<0.0001*	*0.8337*	*0.5651*
	P>F AMix-SMix Estimate	*0.0002*	*0.0002*	*0.0044*	*0.0004*	*0.0010*	*0.4509*	*0.4542*	*<0.0001*	*0.7632*
Cut 2	Alfalfa	8.6963 ab	20.7068 a	35.4371 d	29.4738 c	1.3800 a	0.1961	2.4314 a	0.3142	0.3510
	Alfalfa + Meadow Brome	8.0157 b	19.1354 a	42.1396 c	31.1670 bc	1.2151 ab	0.1920	2.3709 a	0.3806	0.4014
	Meadow Brome	9.9125 a	10.8242 c	59.7200 a	37.8124 a	0.4854 c	0.1795	2.2600 a	0.2381	0.3531
	Sainfoin	6.2242 c	15.2019 b	36.5878 d	32.7351 bc	0.9947 b	0.1862	1.5536 b	0.4243	0.3429
	Sainfoin + Meadow Brome	8.4375 b	13.4430 b	51.0936 b	36.0587 ab	0.7089 c	0.1981	2.4929 a	0.2743	0.3678
	P > F	*0.0006*	*<0.0001*	*<0.0001*	*0.0163*	*<0.0001*	*0.3528*	*0.0003*	*0.1835*	*0.2146*
	SE	*0.4082*	*2.7145*	*2.0612*	*1.7457*	*0.0810*	*0.0079*	*0.1162*	*0.0556*	*0.0213*
	P>F A-S Estimate	*0.0011*	*<0.0001*	*0.6561*	*0.1720*	*0.0038*	*0.3326*	*<0.0001*	*0.1949*	*0.7514*
	P>F AMix-SMix Estimate	*0.4813*	*<0.0001*	*0.0040*	*0.0500*	*0.0005*	*0.5441*	*0.4368*	*0.1868*	*0.2061*

Table 5-9: 2008 plant proximate analysis of five forages in the alfalfa manure treatment at the two harvest dates. All parameters are expressed on a % dry matter basis. Species mixture proximate values were analyzed by species, then mathematically combined on a % dry matter species composition basis.

Cut	Forage	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	Alfalfa	9.6897 a	20.0431 a	43.6612 d	35.7113 c	1.5165 a	0.2141 ab	2.7968 a	0.3332 a	0.3889
	Alfalfa + Meadow Brome	8.5965 b	18.1026 a	54.5793 c	36.6450 c	0.9580 b	0.1875 bc	2.5889 a	0.2664 b	0.4052
	Meadow Brome	6.6534 c	10.3308 c	71.2571 a	43.6562 a	0.2300 c	0.1690 c	2.1989 b	0.0967 d	0.3651
	Sainfoin	6.4530 c	15.3096 b	44.6319 d	38.2203 bc	0.8710 b	0.2384 a	1.8567 c	0.3059 ab	0.3672
	Sainfoin + Meadow Brome	6.4127 c	13.7988 b	63.1713 b	42.2533 ab	0.4813 c	0.2060 abc	2.2060 b	0.1714 c	0.3970
	P > F	*<0.0001*	*<0.0001*	*<0.0001*	*0.0039*	*<0.0001*	*0.0365*	*<0.0001*	*<0.0001*	*0.4213*
	SE	*0.3202*	*2.1293*	*1.9708*	*1.2645*	*0.0816*	*0.0127*	*0.1213*	*0.0167*	*0.0193*
	P>F A-S Estimate	*<0.0001*	*0.0002*	*0.7594*	*0.2048*	*0.0002*	*0.2382*	*<0.0001*	*0.2711*	*0.3955*
	P>F AMix-SMix Estimate	*0.0003*	*0.0031*	*0.0169*	*0.0111*	*0.0021*	*0.3628*	*0.0083*	*0.0017*	*0.7477*
Cut 2	Alfalfa	8.8165 ab	18.0581 a	40.0265 c	32.4303	1.3936 a	0.1989	2.4243 a	0.3178	0.3771
	Alfalfa + Meadow Brome	8.5909 ab	13.6791 b	46.0387 bc	34.5942	1.0952 ab	0.1811	2.4852 a	0.3292	0.3738
	Meadow Brome	9.3778 a	6.1978 d	61.4006 a	38.7525	0.4309 c	0.1962	1.9495 b	0.2151	0.3789
	Sainfoin	6.5586 c	12.2712 b	40.4847 bc	36.4330	1.0723 b	0.1852	1.6589 b	0.3920	0.3435
	Sainfoin + Meadow Brome	7.8347 b	9.6325 c	47.6970 b	34.2122	0.8791 b	0.1832	2.0305 ab	0.3063	0.3968
	P > F	*0.0006*	*<0.0001*	*0.0003*	*0.1519*	*0.0002*	*0.1887*	*0.0132*	*0.1824*	*0.5130*
	SE	*0.3401*	*2.2617*	*2.3064*	*1.8490*	*0.0992*	*0.0072*	*0.1413*	*0.0479*	*0.0232*
	P>F A-S Estimate	*0.0004*	*0.0005*	*0.8949*	*0.1181*	*0.0381*	*0.1302*	*0.0043*	*0.2824*	*0.2732*
	P>F AMix-SMix Estimate	*0.1350*	*0.0011*	*0.6340*	*0.8750*	*0.1431*	*0.8031*	*0.0590*	*0.7350*	*0.4477*

reflected in dry matter species composition data shown in Appendices 8-1 to 8-4.

While there is sporadic evidence of positive manure fertility effects on resident forage nutrient content, a consistent difference between manure sources was not realized at all in the key forage quality parameters of crude protein, NDF, and ADF, nor consistently in calcium, phosphorus, potassium, magnesium, and sodium. This in keeping with the sole published study of the effects of tannin and non-tannin manure origin upon crop availability (Powell and Graber 2009), where two non-tannin containing forages (alfalfa and red clover (*Trifolium pratense*) and birdsfoot trefoil with low (0.51-0.56 % of dietary DM) and high (1.48-1.66% of dietary DM) plant tannin concentrations were fed to dairy cattle. The resultant slurries of feces and urine were band-applied in both spring and spring and fall to soils sown with corn (*Zea mays*). Though the slurries had differing nutritive contents, slurry type had few significant impacts upon soil N, grain N, grain yield, and N uptake. As a result, the researchers were unable to establish a consistent tannin effect upon nutrient availability of dairy manure in the season of or the season following application (Powell and Graber 2009).

Studying plant proximate analysis in the growing season following solid beef cattle manure application at 44.8 t/ha is not sufficient to conclude that bioavailability of manure-derived nutrients differs between sainfoin and alfalfa manure origin.

5.4 Sainfoin tannin concentration

Comparisons on plant tannin concentration were made: 1) across years to establish baseline values for further treatment comparisons; 2) across manure treatments to study the potential differences of manure application and manure origin on tannin production, and 3) between monoculture and mixed species plots in order to characterize intra-specific competitive effects on sainfoin tannin concentration.

5.4.1 2007 vs. 2008 control treatment: non-manured sainfoin condensed tannin concentration

When grown in mixture with meadow brome, condensed tannin (CT) concentration of sainfoin in the second cut at 50-75% bloom was significantly greater ($P < 0.05$) in 2008 (78.0 g/kg) than in 2007 (58.5 g/kg) (Table 5-9). This trend ($P < 0.10$) was also observed in the second cut of sainfoin grown in monoculture (75.1 vs. 56.9 g/kg). There was, however, no discernable difference between 2007 and 2008 first cut sainfoin tannin concentrations. First cut CT concentration in sainfoin monoculture and sainfoin + meadow brome mixtures were 26.49-32.22 g/kg and 30.04-31.26 g/kg, respectively. Second cut sainfoin CT concentration was significantly different ($p = 0.0243$) between 2007 and 2008 when grown in mixture with meadow brome. Condensed tannin concentration increased approximately 25% in 2008, from 58.50 g/kg in 2007 to 78.03 g/kg; a similar trend was also observed in sainfoin monoculture, however, high variability in CT concentrations between repetitions mitigated significant statistical comparison.

Table 5-10: Year comparison of condensed tannin (CT) concentration (g kg-1) in sainfoin grown in monoculture and in mixture with meadow brome in 2007 and 2008.

Cut	Year	Sainfoin CT (g kg $^{-1}$) by Forage Stand	
		Sainfoin Monoculture	*Sainfoin in Mixture*
Cut 1	2007	32.2221	31.2628
	2008	26.4985	30.0408
	P > F	*0.5308*	0.7952
	SE	*6.2312*	2.5281
Cut 2	2007	56.9314	58.5015 b
	2008	75.1369	78.0285 a
	P > F	*0.0891*	0.0243
	SE	*9.0241*	8.5912

The almost twofold increase in sainfoin tannin concentration from first to second harvest was not anticipated. The sole previous tanniniferous species survey data in Manitoba (Knox et al., unpublished) found that vegetative and mature sainfoin contained 58.7 g/kg and 33.7 g/kg CT, respectively, however, this survey did not examine the effect of harvest date upon plant tannin concentration at similar stages of maturity. Higher second cut tannin values were previously observed by McMahon et al. (1999), however, callous explant study by Lees et al. (1995) showed significant catabolism of leaf vacuole tannin deposits prior to maturation and through senescence. Whole-plant sainfoin levels observed in the present study are are substantially greater than the 28 g/kg previously reported for whole-plant sainfoin hay (McMahon et al.1999), though maturity at harvest was not reported. Given that in the present study, both harvests in 2007 and 2008 occurred while sainfoin was primarily vegetative, the CT concentrations of non-manured plots in both years was higher than anticipated. These findings may have been compounded by a previously unstudied effect of harvest upon sainfoin CT concentration,

where the significant disturbance (via hand or mechanical means) increases CT concentration in subsequent regrowth.

The observed increase in temperature and uneven seasonal precipitation distribution in 2007 may have played a role in sainfoin tannin production (see Table 5-1). Average temperatures from May 1 – August 31 at Carman, MB. were approximately 15% greater in 2007 than in 2008. Cumulative precipitation for this period was 60.8mm, or 22.4% greater in 2007. In spite of lower total precipitation in 2008, below-average temperatures along with more uniform late-season precipitation may have promoted increased vegetative growth. The higher sainfoin CT concentrations seen during the more moderate growing season of 2008 contrast with the previously published concept of increased plant CT investment during stressful or growth-limiting conditions (Barry and Duncan 1984; Anuraga et al. 1993).

Higher whole plant sainfoin tannin concentration at later maturities supports the observations by Koupai-Abyazani (1993b), who showed increasing leaf condensed tannin concentrations with maturity when measured with HCl-butanol assays.

Field observations showed little lower leaf senescence at harvest in either first or second cuts, which would potentially decrease the leaf to stem ratio of the forage sample. However, since plant partitioning measurements were not a component of this study, this factor cannot be confirmed. Since sainfoin plants were harvested and stored as fresh, frozen material prior to tannin analysis, little if any leaf loss occurred during harvest, transport, or processing, thus experimental whole-plant CT concentrations are reflective of the forage material immediately prior to harvest.

Results from the non-manured year comparison support increased whole plant sainfoin CT concentration in later harvests, and substantial variation in second cut CT concentration between years.

5.4.2 2008 sainfoin tannin concentration, comparing manure origin and application in sainfoin monoculture and sainfoin + meadow brome mixtures

In both the sainfoin monoculture and sainfoin + meadow brome mixture forage treatments, application of either alfalfa or sainfoin-derived solid beef cattle manures in the fall of 2007 did not significantly affect sainfoin tannin concentrations in either 1st or 2nd cuts of 2008 (Table 5-10). There was a trend in the second cut of sainfoin in mixture with meadow brome ($p = 0.0762$), where application of either alfalfa or sainfoin manure

Table 5-11: Sainfoin condensed tannin concentration (g/kg) in three manure treatments when grown in monoculture and in mixture with meadow brome in 2008.

Cut	Manure Treatment	Sainfoin CT (g kg^{-1}) by Forage Stand	
		Sainfoin Monoculture	*Sainfoin in Mixture*
Cut 1	Alfalfa Manure	26.3358	32.8525
	Control (no manure)	26.4985	30.0408
	Sainfoin Manure	29.4468	35.0239
	P > F	*0.7962*	*0.6755*
	SE	*7.1899*	*5.4580*
	P>F A-S Estimate	*ns*	*ns*
Cut 2	Alfalfa Manure	73.2000	87.9844
	Control (no manure)	75.1369	78.0285
	Sainfoin Manure	73.9783	99.6000
	P > F	*0.9636*	*0.0762*
	SE	*8.0731*	*10.6146*
	P>F A-S Estimate	*ns*	*ns*

increased sainfoin tannin concentration (87.98 and 99.60 g/kg CT respectively) over the non-manured control (78.03 g/kg CT). A similar trend was not apparent in the sainfoin monoculture.

The lack of a significant difference between alfalfa-derived beef cattle manure and the non-manured control in 2008 indicates that any fertility effects from the manure may not factor into the mechanisms of tannin production in sainfoin. The influence of soil fertility upon plant tannin concentration is inconclusive in published literature. Previous research on CT-producing *Lotus* species by Lowther et al. (1987) concluded that soil nitrogen fertility status did not influence plant CT concentration, while Briggs et al. (1990) showed that inorganic nitrogen fertilization modestly increased plant CT concentration over a nitrogen-depleted control. Conversely, both Barry and Forss (1983) and Barry and Duncan (1984) found that poor soil fertility induced higher plant tannin concentrations. This range of fertility effects on forage CT concentration in the literature illustrates a need for deeper understanding of both CT biosynthetic pathways and their regulatory measures within both the plant and its ecosystem.

Additionally, there was lack of significance between sainfoin-derived beef cattle manure and the non-manured control, as well as in estimates between the two manure types. Since the known non-tannin containing manure did not impact tannin production in resident sainfoin forages, the study lacked a non-tannin containing, manured benchmark from which to evaluate potential "tannin effects" of sainfoin-derived manure on tannin production in sainfoin *in vivo*. Forest research comparisons of fertility (Kraus et al. 2004) and the presence of endogenous tannins on tannin production and nutrient cycling (Northup et al. 1998) were thus not comparable with the present study.

5.4.3 Sainfoin condensed tannin (CT g kg^{-1}) concentration in 2008, comparing competitive effects of sainfoin in mixture vs. monoculture across manure application and manure origin

Sainfoin CT concentration was affected by the presence of a non-tannin producing competitive grass species in only one instance (Table 5-11). The second cut of the sainfoin-manured treatment in 2008 showed a significant difference ($P < 0.05$) between sainfoin grown in a mixture with meadow brome (99.60 g/kg CT) compared to sainfoin in a monoculture (73.89 g/kg).

In seven out of eight comparisons, sainfoin in mixture with meadow brome produced higher concentrations of condensed tannins versus sainfoin grown in pure stands. Though these differences were statistically insignificant, they are in agreement with the sole study of interspecific competition on tannin production in forages (Wen et al. 2003), though the forage stand differences were often dwarfed by variability of tannin concentration within treatments.

Table 5-12: Condensed tannin concentration (g/kg) of sainfoin comparing monoculture and mixture stand types in 2007 and 2008 control and manure treatments.

Cut	Forage Stand Type	Sainfoin CT (g kg^{-1}) Year and Manure Treatment			
		2007	2008	2008	2008
		Control	*Alfalfa Manure*	*Control*	*Sainfoin Manure*
Cut 1	Sainfoin in Mixture	31.2627	32.8525	30.0408	35.0239
	Sainfoin Monoculture	32.2221	26.3358	26.4985	29.4468
	P > F	*0.7410*	*0.2196*	*0.6659*	*0.4268*
	SE	*1.5499*	*5.5784*	*6.5434*	*6.0602*
Cut 2	Sainfoin in Mixture	58.5015	87.9844	78.0285	99.6000 a
	Sainfoin Monoculture	56.9314	73.2000	75.1369	73.9783 b
	P > F	*0.4217*	*0.1498*	*0.8273*	*0.0471*
	SE	*7.8247*	*8.6807*	*9.6962*	*9.8689*

The positive effect that the presence of meadow brome in mixture with sainfoin had upon sainfoin tannin concentration may have been due to previously-hypothesized

changes in microclimate or light interception (Wen et al. 2003). This cannot be stated conclusively, as neither parameter was within the scope of this study. Alternatively, it is plausible that the additional competitive stress that the non-leguminous meadow brome placed upon the nutrient availability within the mixture created a less fertile environment for sainfoin versus that grown in monoculture. This effect of competitive stress on resource availability is in keeping with previously cited research by Barry and Forss (1983) and Barry and Duncan (1984), who observed a reduction in plant tannin concentration once environmental limitations of low fertility and pH were removed from a forage production scenario.

5.4.4 2007 and 2008 whole plot (sainfoin + meadow brome) condensed tannin concentration

In 2007, manure-free sainfoin + meadow brome plots produced 15.87 and 28.36 g/kg CT in first and second cuts, respectively, based upon the CT production in sainfoin multiplied by the % dry matter sainfoin in the species mixture. Though these values differed slightly in 2008, no significant year difference (2007 vs. 2008) (Table 5-12) or manure difference in 2008 (alfalfa manure, non-manured control, sainfoin manure) (Table 5-13) was established, leading to the conclusion that neither year, application of solid beef cattle manure, nor manure origin affects tannin concentration of sainfoin + meadow brome mixtures. The significant difference in sainfoin CT concentration when grown in mixture with meadow brome under sainfoin manure was eliminated when comparing both sainfoin and meadow brome together in whole plot analysis.

When sainfoin was grown in mixture with meadow brome, the resulting forage sward exceeded the minimum tannin concentration recommendations for bloat safety

previously proposed by Li et al. (1996) of 0.1-0.5% DM, and later by McMahon et al. (1999) of 0.35-0.46% DM by several orders of magnitude. Given these high tannin values in the sainfoin forage component, these bloat safe levels could theoretically be achieved with less sainfoin per unit of meadow brome.

Table 5-13: Year comparison of the mathematical condensed tannin concentration (g/kg) of sainfoin + meadow brome grown in mixture in a non-manured control treatment.

Cut	Year	CT (g kg^{-1})
Cut 1	2007	15.8670
	2008	10.8999
	P > F	*0.2545*
	SE	*2.7724*
Cut 2	2007	28.3587
	2008	49.6719
	P > F	*0.0856*
	SE	*6.6340*

Table 5-14: Manure treatment comparison of the mathematical condensed tannin concentration (g/kg) of sainfoin + meadow brome grown in mixture.

Cut	Manure Treatment	CT (g kg^{-1})
Cut 1	Alfalfa Manure	11.2377
	Control (no manure)	10.8998
	Sainfoin Manure	10.0055
	P > F	*0.7863*
	SE	*4.0011*
	P>F A-S Estimate	*ns*
Cut 2	Alfalfa Manure	31.9493
	Control (no manure)	49.6719
	Sainfoin Manure	40.0169
	P > F	*0.3110*
	SE	*8.4253*
	P>F A-S Estimate	*ns*

5.4.5 Stepwise regression of soil nutrient and plant proximate analysis parameters on sainfoin condensed tannin concentration

Given the lack of a predictive framework to model condensed tannin by leguminous forages under typical commercial management scenarios, three multiple regression stepwise analyses on sainfoin condensed tannin concentration were conducted to guide future investigation into sainfoin tannin production. These regressions were: 1) including all soil physical and chemical analyses on associated sainfoin condensed tannin concentration, 2) including all plant proximate analysis parameters on sainfoin condensed tannin concentration, and 3) combining both plant and soil analyses upon associated sainfoin condensed tannin concentration. These regressions were each conducted across both 2007 and 2008 years, and included all forage (monoculture and mixture) and manure (no manure, sainfoin manure, alfalfa manure) treatments. The aim of this analysis was to correlate the greatest range of experimental soil fertility and forage quality values to sainfoin CT concentration across the four harvest dates.

1. Plant proximate analysis stepwise regression revealed that plant NDF and phosphorus content were the only two out of 10 parameters to be entered as significant variables in the model, contributing to a combined partial R^2 of 0.6740 (0.5481 and 0.1259 for NDF and P, respectively) (Table 5-14). Both NDF and plant P were strongly ($P < 0.0001$) and negatively (-0.7404 and -0.7209, respectively) correlated with sainfoin CT concentration.
2. Soil nutrient stepwise regression showed that out of 11 parameters, soil nitrogen content from 15-60cm was the only significant variable entered into the model,

contributing a partial R^2 of 0.0779 (Table 5-15). This variable was negatively correlated (-0.2749) with sainfoin CT concentration.

3. Combined soil nutrient and plant proximate stepwise regression, entering all 21 parameters, showed that the aforementioned plant NDF and P again were entered into the model, contributing to a combined partial R^2 of 0.6740, and that soil K in the 0-15cm horizon was the sole other significant variable, with a partial R^2 of 0.0234 (Table 5-16).

The quantitative predictive ability of stepwise regression is limited when containing many interrelated and interactive parameters, and given the inclusive P value (0.15), the present model should be regarded as preliminary evidence. The model entries explained above suggest that tannin production in sainfoin is regulated in part by mechanisms associated with fibre accumulation and plant phosphorus content. Fibre accumulation has been shown to increase with maturity in alfalfa (Cassida et al. 2000) due to a decreased plant investment in vegetative growth over time. The negative correlation shown between NDF and plant tannin concentration may therefore insinuate that growth factors encouraging vegetative development, such as optimal soil moisture (Peel et al. 2004), moderate air temperatures (Bolger and Matches 1990; Kallenbach et al. 1996), and adequate soil fertility (Meyer 1975) may increase plant tannin production. A variation of this theory was first proposed by Lees et al. (1993, 1995) in that plant investment in secondary metabolites such as condensed tannins may be a “luxury” biosynthetic pathway that is stimulated by favourable growth conditions.

Table 5-15: Summary of stepwise regression of all sainfoin plant measurements on condensed tannin concentration across all treatments and years.

Step	Entered	Variable Removed	Variable Vars In	Number R-Square	Partial R-Square	Model C(p)	F Value	P > F
1	NDF		1	0.5481	0.5481	26.2467	75.21	<.0001
2	Plant Phosphorous		2	0.1259	0.6740	4.2260	23.55	<.0001
3	Plant Magnesium		3	0.0146	0.6886	3.4336	2.82	0.0984
4	Plant Ash		4	0.0129	0.7015	2.9707	2.55	0.1156

Table 5-16: Summary of stepwise regression of all sainfoin soil nutrient parameters on condensed tannin concentration across all treatments and years.

Step	Entered	Variable Removed	Variable Vars In	Number R-Square	Partial R-Square	Model C(p)	F Value	P > F
1	Soil Nitrogen 15-60cm		1	0.0779	0.0779	-5.4069	5.24	0.0255

Table 5-17: Summary of stepwise regression of all sainfoin plant measurements and soil nutrient parameters on condensed tannin concentration across all treatments and years.

Step	Entered	Variable Removed	Variable Vars In	Number R-Square	Partial R-Square	Model C(p)	F Value	P > F
1	NDF		1	0.5481	0.5481	21.0548	75.21	<.0001
2	Plant Phosphorous		2	0.1259	0.6740	0.4802	23.55	<.0001
3	Soil Potassium 0-15cm		3	0.0234	0.6974	-1.7133	4.63	0.0354
4	Soil OM% 0-15cm		4	0.0167	0.7141	-2.7141	3.45	0.0682

These assumptions are likely specific to time and species studied; similar work in natural systems research (Fierer et al. 2001; Hättenschwiler et al. 2003; Kraus et al. 2004) has proposed that tannins are a stress-mediated plant product, and their production is a function of competitiveness in resource-limited environments. Further investigation of sainfoin tannin dynamics within the context of highly productive agricultural systems is necessary to refine *in vivo* understanding of this and other tanniniferous forage species.

6.0 SYNTHESIS

6.1 Overall purpose

To investigate potential tannin-related effects upon nutrient bioavailability of sainfoin-derived solid beef cattle manure, as measured through forage biomass yield, soil nutrient status, and plant proximate analysis in the year following manure application.

To identify tannin-related effects of sainfoin-derived solid beef cattle manure upon condensed tannin production in resident sainfoin forages.

To determine if tannin concentration in sainfoin is altered as a function of competitive stress by the presence of meadow brome in a species mixture versus a pure sainfoin stand.

6.2 Summary of findings

Whole-plant sainfoin CT concentration was variable throughout the experiment; intra-plot variation within harvest dates often exceeded treatment differences. Application of either sainfoin-derived or alfalfa-derived stockpiled solid beef cattle manures did not significantly and consistently affect sainfoin CT levels in either a monoculture or species mixture with meadow brome. Based upon concurrent observations of plant proximate analysis, soil nutrient status, and forage biomass production as indicators of manure bioavailability, it appears as though the experimental

rate of 44.8 t/ha of solid beef manure contained insufficient available nutrients to impact resident forages in the growing season following application, regardless of origin. The absence of C:N ratio data of original feedstuffs, manures, and resident plant material has limited the ability to more accurately characterize the driving factors behind gross nutrient cycling in tannin-containing tame forage crops. There were no additional non-fertility, tannin effects of sainfoin-derived manure upon tannin production in resident sainfoin forages, and growing sainfoin in a species mixture did not increase sainfoin tannin concentration. Environmental differences between years, and temporal differences within a year appear have been shown to exert greater influence upon sainfoin CT concentration than management strategies.

Summarily, the voluminous data set generated by the present study yielded few consistent significant conclusions about the treatments, and none of the experimental hypotheses were proven true. Similar multi-disciplinary research on plant tannins, beef cattle manure, and tame forage agronomy was without precedent in the literature. When the present study was accompanied by an associated study investigating enteric methane emissions from sainfoin-fed beef cattle (Bouchard, unpublished), condensed tannins were represented for the first time in all segments of a forage-based livestock production system. These baseline values will provide a comparative framework for tannin-related agronomic research in the Canadian prairies.

6.3 Implications in Canadian livestock production systems

The present state of knowledge of tannin interactions in ruminant livestock production has largely been the providence of research outside of North America, in livestock species other than *Bos Taurus*, using forages other than sainfoin. The present study has provided a data set examining the interactions of soil, plant, and manure using domesticated tannin-containing forages in the northern Great Plains.

The lowest sainfoin CT concentration expressed within any single sample of sainfoin or sainfoin + meadow brome exceeded the minimum recommended range of 0.1-0.5% DM (1.0-5.0 g/kg) for bloat safety in leguminous forages (Li et al. 1996; McMahon et al. 1999). The narrow range of first cut sainfoin monoculture CT across all years and manure treatments (26.5-35.0 g/kg DM) falls within the range where anthelmintic effects have been shown in sheep and goats (Paolini et al. 2003; Athanasiadou et al. 2005; Hoste et al.). The substantial increase in CT concentration in the second cut of both 2007 (56.9-58.5 g/kg) and 2008 (73.2-99.6 g/kg) was unexpected and above previously published whole-plant CT values. The positive postingestive effects of these higher sainfoin CT concentrations upon feed efficiency, digestive passage rates, and dry matter intake have not been studied in any ruminant species, and thus any assumptions of CT effects within the feeding value of sainfoin or sainfoin + meadow brome at levels observed in the present study are purely speculative.

Proliferation of sainfoin into the northern Great Plains as either a hayed or grazed perennial legume has been hampered by several agronomic factors. An inherently large

seed size, coupled with the high seeding rate required for optimal stand establishment places sainfoin at a cost disadvantage relative to alfalfa (Goplen et al. 1991). Compounding these high initial costs are published concerns of stand productivity, post-harvest re-growth, and persistence in regions where soil moisture is adequate to allow for more than one cut. Previously reported poor competitive ability with weeds and persistence within forage species in mixtures limits sainfoin recommendations to use in short-term forage stands. As evidenced in the biomass measurements of the 2nd and 3rd seasons after establishment, such stand deterioration was not apparent in terms of yield or species composition in the present study. Dry matter biomass yields in 2007 and 2008 across all manure treatments re-affirm that sainfoin possesses early season growth potential equal to or exceeding that of alfalfa, though this benefit is offset by poor mid-season re-growth leading to lower second cut biomass under a two-cut hay harvest schedule.

While attempting to quantify the effects of tannin-containing forage diets upon the fertility value of stockpiled solid beef manure, it was observed that a fall-applied rate of 44.8 t/ha did not significantly impact forage biomass, primary forage nutritive parameters of CP, NDF, and ADF, or residual fall soil test values in the subsequent year, regardless of manure origin. The lack of a significant fertility effect difference from either sainfoin or alfalfa-derived manure versus a non-manured control mitigated comparison of nutrient bioavailability between the manures. Recent work evaluating the fertilizer replacement value of solid beef cattle manure in Manitoba in mixed native and tame grass pastures has shown that application rates containing three times the assumed

plant-available nitrogen/hectare of the present study (75kg/ha vs. 21.8-25.2 kg/ha) showed a significant effect on forage yield at a mid-season harvest date (Kumaragamage et al., unpublished). They noted that this forage yield response was statistically similar to a urea fertilizer treatment at the same nitrogen rate. It is acknowledged that bioavailability of stockpiled manures are by nature extremely variable and difficult to predict, however, this local comparison is evidence that higher rates of solid beef manure may be required to positively impact forage yields in the following season in Manitoba.

6.4 Recommendations for future research

If the inclusion of tannins in livestock systems is deemed legitimately valuable for either environmental or production-related characteristics, a suitable delivery mechanism is required. The industrial paradigm which dominates North American livestock production would demand that this mechanism take the form an easily-integrated commercial feed ingredient – perhaps an organic extract - and indeed, this approach is reflected in recent studies (Grainger et al. 2009; Wang et al. 2009; Krueger et al. 2010). Tannins may play a role in increasing the efficiency or limiting the environmental footprint of an industrially-motivated, low-cost livestock production complex. Alternatively, tannins may be "applied" via management-intensive ruminant grazing of tannin-containing tame forages. Such perennial systems acknowledge agriculture's central role in functional environmental stewardship via a less anthropocentric approach to sustainable and safe food production. Selection for either application of tannin

research will reflect the underlying directions and concerns of the agricultural scientific community.

Present recommendation of common tannin-containing forages, such as sainfoin, birdsfoot trefoil, and alsike clover (*Trifolium hybridium*,) to livestock producers is limited by inferior agronomic and nutritive qualities to alfalfa and alfalfa-grass mixtures. Given that sainfoin exhibited productivity potential rivalling or exceeding that of alfalfa in the present study, and has previously been identified as containing the optimal tannin profile for positive nutritive effects in ruminants (McAllister et al. 2005), it appears the most likely candidate for development. Based upon published criticisms of sainfoin, efforts should be directed at improving post-harvest regrowth, winter hardiness, response to drought stress, and competitive abilities with companion crops. Without significant investment and progress in sainfoin development, it appears at present that tannin integration in forage-based livestock production will be initiated by initiating or increasing expression of tannin production in commonly-used legumes, especially alfalfa, through either conventional breeding or genetic manipulation (Ray et al. 2003; Yu et al. 2009).

The whole of tannin knowledge in ruminants is based on a loosely-related global collection of plant and animal studies spanning several disciplines, often using non-standardized methods, locally-relevant species, and "adapted" operating procedures. The resultant body of work has yielded many favourable benefits to including tannins in livestock production systems, however, if the study of tannins in agriculture is to mature beyond its presently exploratory nature rife with *qualitative* comparisons and site-specific

recommendations, these methodological and procedural discrepancies need to be eliminated. Standardization of chemical and biological protocols through an acknowledged body, such as the Association of Official Analytical Chemists, would allow for reasonable *quantitative* experimental comparisons across time and place, even if, as suggested by Mueller-Harvey (2006), several simultaneous analytical techniques are required to provide biologically meaningful information to nutritionists, veterinarians, plant breeders, and agronomists. Only when such standardized methods are synchronized with comprehensive agronomic and proximate screening of known tannin-producing forage species will predictive, systems-level modelling of tannin activity in both plant and animal be possible. Substantial time and resources will be devoted to participating in a proverbial game of "pin the tail on the donkey" until a basal understanding of tannin chemical and biological activity *in vivo* is established. Resources from analogous studies in forest ecosystems, as previously reviewed, may expedite this process.

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8.0 APPENDICES

Appendix 8-1: Condensed tannin quantification procedure used in the present study.

Standard Operating Procedure from Dr. Yuxi Wang's Lab by Zhong J. Xu, 2005-08-18

Text prepared by Janice Haines, modified by Robert Kazuk

Caution and Safety

- Always consult the MSDS sheets before preparing reagents especially when using chemicals that have hazard labels. Exercise great caution in handling the chemicals. All reagents are very flammable. The chemicals used in Reagent B are very toxic. The assay reagent may cause very bad headaches, strong odours and is very flammable as well.
- All steps should be carried out in a fume hood if possible. Use only screw–cap centrifuge tubes for centrifugation.
- Wear gloves, safety glasses, a respirator and a lab coat when required
- Always use appropriate warning signs and labels on all reagent and waste bottles
- Always dispose of waste according to local laws and regulations
- Do not throw hazardous waste down the drain or into the regular garbage system

Chemical and Suppliers

Acetone	UN 1090, EM
Diethyl Ether	B14993, BDH
Ascorbic Acid	A-7506, Sigma
Butanol	A399-1, Fisher
Hydrochloric Acid (36%)	A144-212, Fisher
Trizma Hydrochloride	T7149, Sigma
Sodium lauryl sulphate (Sodium dodecyl sulphate)	S529, Fisher
2-mercaptoethanol	M6250, Sigma

Tannin Standard Material – Prepared preferably from the same species as the experimental material. In absence of such material, commercially-available condensed tannin extracts may be used.

Equipment:

- boiling flasks with round glass necks for use on a rotary evaporator
- Pyrex™ screw cap 25 x 200 mm 75 ml round bottom tubes with PTFE-faced rubber lined cap
- Test tube racks capable of withstanding boiling
- Rotary evaporator with 45°C water bath
- 50 ml volumetric flasks to store sample extractions
- Centrifuge and rotors capable of spinning 50 ml tubes at 3500 X g
- Centrifuge and rotors capable of spinning 15 ml tubes at 16000 X g
- Boiling water bath
- Spectrophotometer
- Assorted beakers
- 10 and 20 ml pipettors (Rainin is preferred Mfg.)

Disposables:

50 ml disposable conical base screw cap tubes - polycarbonate is not suitable for acetone (2 x2 duplicates/sample)

- 15 ml screw cap tubes (3 x 2 duplicates /sample)
- cuvettes
- Pasteur pipettes and bulbs
- 10 and 20 ml pipette tips

Solutions:

Extraction Reagent A1

- Acetone 700 ml/L
- Water 300 ml/L
- Ascorbic Acid 1.0 g/L

Make up using a volumetric flask

Make 42 ml per sample (14 ml x 3 treatments)

Extraction Reagent A2

- Diethyl ether
  Requires 21 ml per sample (7 ml X 3 treatments)

Extraction Reagent B – pH 8.0

- 10 mM Trizma Hydrochloride 1.576 g/L
- Sodium lauryl sulphate (SDS) 10 g/L
- 2-mercaptoethanol (caution-very toxic) 50 g/L

Make up using a volumetric flask and deionised water

Adjust pH using either HCl to lower the pH or NaOH to raise the pH.

Requires 50 ml of solution per sample and standards.

Assay Reagent

- Hydrochloric acid 5 ml/L
- Butan-1-ol 95 ml/L

Make up using a volumetric flask.

Requires 6 ml of solution per sample and standards.

Standard

Prepare the standards in either deionised water for use with the soluble CT assay or with Reagent B for use with the protein-bound assay and the fiber-bound assay. Use a 4 decimal place scale to weigh the tannin 1g/L (1mg/ml). Prepare the standard curve by dispensing the 1mg/ml stock into labelled 15 ml screw cap tubes to make a range of 5 concentrations as follows.

Concentration	ml of stock (1mg/ml)	ml of water or Reagent B
1 mg/ml	2	0
0.75	1.5	0.5
0.50	1.0	1.0
0.25	0.5	1.5
0.125	0.25	1.75

Vortex to mix and then pipette 1 ml of each into another labeled series of tubes to act as the duplicates.

Note: Purified tannin is a precious commodity. Make only enough stock solution to run the assay. If you are running assays on consecutive days it may be possible to store the stock soln at 4°C if it is kept in the dark to prevent oxidation from light exposure. Use a screw cap tube or vial and wrap it with foil to prevent oxidative degradation.

Sample Preparation

Freeze drying is preferable to oven drying. Grind to pass through a 1 mm screen.

Extraction Procedures:

Step 1: Extraction of Soluble CT

1. Using a 4 decimal place scale weigh out 500 mg of each plant sample in duplicate into a labeled 50 ml tube. Record the exact weight of each sample
2. Add 14 ml of extract reagent A1 to each tube. Add 7 ml of reagent A2 to each tube. Always add reagent A1 first. Vortex and leave the sample tubes sitting at room temperature for 20 minutes. Vortex every 5 minutes.
3. Centrifuge at 3500g for 20 minutes. Transfer supernatant to a labeled 75 ml glass tube. Save the residue in the 50 ml tube from step 1-1 for Step 2
4. Repeat steps 2 and 3 two more times adding the supernatant to the tube. . Refrigerate until you are ready to do the extraction.
5. The supernatant should separate into 2 phases, one green and the other yellow/orange. If this does not occur, add a small amount of diethyl ether to the 75 ml glass tube and shake. Cap and mix well.
6. Aspirate and discard the top layer of solvent (ether and acetone). Use the 20 ml pipettor to remove the ether at first. Switch to the 10 ml pipettor for more control removing the last bit. Do not remove the lower aqueous phase. The remaining ether and acetone will be removed from the aqueous layer next.
7. Transfer the supernatant (bottom layer of solvent) into the boiling flasks. Rinse the 75 ml tube with 1 ml of deionised water and transfer to the boiling flask.
8. Remove the trace solvents by rotary evaporation at 45°C for 4 minutes. Complete evaporation is important. Waft the fumes from the top of the boiling flask toward you. If there is a strong odour of ether more time is

needed. More time is required depending on the rotary evaporator and the vacuum suction. Evaporation times in the present

9. Transfer the supernatant into a 50 ml volumetric flask. Rinse two more times with small amounts of deionised water. Transfer the rinses to the 50 ml flask. Bring to volume using deionised water. Use 100 ml flasks for highly condensed tannin samples. Mix well
10. Transfer 5 ml of aqueous extract to labelled 15 ml centrifuge tubes. Centrifuge at 16000g to remove non-tannin debris for the late assay. Store at 4° until ready to assay.
11. These extracts will be assayed against the blank and standards prepared in water.
12. The hazardous waste solutions must be stored in approved plastic jugs and removed by the Safety Office.

Step 2: Extraction of protein- bound CT

1. Add 15 ml of Reagent B to solid residue from Step 1-3. Cap tightly. Vortex and leave the tubes sitting at room temperature for 20 minutes. Vortex every 5 minutes
2. Place the tubes into a constant level boiling water bath for 45 minutes.
3. Remove the tubes from the bath and cool to room temperature with cold water in the sink. Centrifuge at 16000g for 10 minutes. Transfer supernatant into 50 ml disposable tubes.
4. Repeat the extraction with Reagent B, steps 1-3, combining the supernatants in the 50 ml tube. Save the pellets for Step 3 capping tightly and store at 4°C
5. Transfer the supernatant into a 50 ml volumetric flask. Rinse the tube twice with reagent B and pour the rinses into the flask. Make up to volume using reagent B. Use 100 ml flasks for highly condensed tannin samples.
6. Mix well and transfer 5 ml of aqueous extract to the 15 ml centrifuge tubes. Centrifuge at 16000g to remove non-tannin debris.
7. This extract will be assayed against standards prepared in Reagent B. Store at 4°C, if necessary, until ready to assay.

Step 3: Fibre-bound CT

1. Solid residue from step 2 is analyzed for CT directly by adding 30 ml of Assay Reagent and 3 ml of Reagent B. Vortex.
2. This CT will be assayed and determined against standards prepared in reagent B and blanked spec with Assay reagent. Store at 4°C, if necessary, until ready to assay.

Assay Procedure

1. Prepare the standard curve as described in the solutions section.
2. Pipette 1 ml of sample into the 15 ml screw cap tubes
3. Add 6 ml of Assay Reagent (Butanol-HCl) to each of the samples and standards.
4. Vortex
5. Boil all of the tubes in a constant level boiling water bath for 75 minutes
6. Cool in an ice bath
7. Read at 550nm on a spectrophotometer. Record sample ID and absorbance. If a standard or a sample yields values too high on the spec to produce linearity (i.e. over 1.000), dilute them with the appropriate agent, then read again and record the dilution factor
8. Plot the absorbance vs. concentration to construct the calibration curve and determine the X coefficient. To calculate sample concentration divide the absorbance by the X coefficient.

Appendix 8-2: Alfalfa + meadow brome forage mixture breakdown by species: year comparison (2007 vs. 2008) of the control (non-manured) treatment.

Cut	**Year**	**Forage Stand DM Biomass (kg ha $^{-1}$) and Species Composition (%DM)**			
		Alfalfa		Meadow Brome	
		Biomass	*Species Composition*	*Biomass*	*Species Composition*
Cut 1	2007	2986	0.8049	701 b	0.1952
	2008	3915	0.7243	1345 a	0.2757
	P > F	*0.1278*	*0.1637*	*0.0346*	*0.1637*
	SE	*545*	*0.0659*	*220*	*0.0659*
Cut 2	2007	2547	0.9574	112	0.0427
	2008	2761	0.8445	512	0.1555
	P > F	*0.6659*	*0.1272*	*0.1098*	*0.1272*
	SE	*292*	*0.0369*	*124*	*0.0369*

Appendix 8-3: Sainfoin + meadow brome forage mixture breakdown by species: year comparison (2007 vs. 2008) of the control (non-manured) treatment.

Cut	**Year**	**Forage Stand DM Biomass (kg ha $^{-1}$) and Species Composition (%DM)**			
		Sainfoin		Meadow Brome	
		Biomass	*Species Composition*	*Biomass*	*Species Composition*
Cut 1	2007	1987	0.5124	1841	0.4876
	2008	1900	0.3464	3764	0.6536
	P > F	*0.3824*	*0.1362*	*0.1702*	*0.1362*
	SE	*429*	*0.0764*	*655*	*0.0765*
Cut 2	2007	851 b	0.5014	712	0.4959
	2008	1846 a	0.6458	1006	0.3543
	P > F	*0.0062*	*0.2362*	*0.3292*	*0.2362*
	SE	*291*	*0.0983*	*177*	*0.0983*

Appendix 8-4: Alfalfa + meadow brome forage mixture breakdown by species: manure treatment comparison in 2008.

Cut	Year	Forage Stand DM Biomass (kg ha $^{-1}$) and Species Composition (%DM)			
		Alfalfa		Meadow Brome	
		Biomass	*Species Composition*	*Biomass*	*Species Composition*
Cut 1	Alfalfa Manure	3118 b	0.6360	1755	0.3640
	Contol (no manure)	3915 a	0.7243	1345	0.2757
	Sainfoin Manure	3426 ab	0.6166	2018	0.3834
	P > F	*0.0419*	*0.1583*	*0.3259*	0.1583
	SE	*724*	*0.1136*	*527*	0.1136
	P>F A-S Estimate	*0.2464*	*ns*	*ns*	*ns*
Cut 2	Alfalfa Manure	2516	0.2086	649	0.2086
	Contol (no manure)	2761	0.1555	512	0.1555
	Sainfoin Manure	2241	0.1966	487	0.1966
	P>F	*0.6318*	*0.7358*	*0.7668*	*0.7358*
	SE	*466*	*0.0482*	*152*	*0.0482*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*

Appendix 8-5: Alfalfa + meadow brome forage mixture breakdown by species: manure treatment comparison in 2008.

Cut	Manure Treatment	Forage Stand DM Biomass (kg ha $^{-1}$) and Species Composition (%DM)			
		Sainfoin		Meadow Brome	
		Biomass	*Species Composition*	*Biomass*	*Species Composition*
Cut 1	Alfalfa Manure	1890	0.2958	4748	0.7042
	Contol (no manure)	1901	0.3464	3764	0.6536
	Sainfoin Manure	2062	0.2899	4437	0.7101
	P > F	*0.8416*	*0.5841*	*0.5367*	*0.5841*
	SE	*589*	*0.0856*	*779*	*0.0856*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*
Cut 2	Alfalfa Manure	908 b	0.3744	1434	0.6256
	Contol (no manure)	1846 a	0.6458	1006	0.3543
	Sainfoin Manure	905 b	0.4658	902	0.5342
	P > F	*0.0037*	*0.1667*	*0.3202*	*0.1667*
	SE	*310*	*0.1169*	*223*	*0.1169*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*

Appendix 8-6: Comparison of the proximate analysis of 2007 alfalfa grown in a monoculture and in mixture with meadow brome.

Cut	Forage Stand	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	w/ Meadow Brome	8.0075	15.6827	48.1721	38.5923	1.5288	0.1933	1.8313	0.3672	0.4042
	Alfalfa Monoculture	8.3248	15.5790	48.3578	39.4727	1.5132	0.1958	2.0558	0.3257	0.4031
	P > F	*0.1450*	*0.8700*	*0.8880*	*0.3369*	*0.8922*	*0.8424*	*0.1393*	*0.3682*	*0.9698*
	SE	*0.1438*	*0.6032*	*1.5903*	*1.5731*	*0.0973*	*0.0128*	*0.0821*	*0.0272*	*0.0150*
Cut 2	w/ Meadow Brome	7.6809	17.3332	46.6599	36.6654	1.1683	0.1800	1.9124	0.3740	0.4589
	Alfalfa Monoculture	7.7464	16.4754	47.5784	38.2404	1.3358	0.1784	1.9172	0.3462	0.4532
	P > F	*0.8736*	*0.1998*	*0.7518*	*0.2442*	*0.4215*	*0.6210*	*0.9895*	*0.6139*	*0.7936*
	SE	*0.2132*	*0.4575*	*1.7318*	*1.1064*	*0.1089*	*0.0066*	*0.2358*	*0.0257*	*0.0262*

Appendix 8-7: Comparison of the proximate analysis of 2007 sainfoin grown in a monoculture and in mixture with meadow brome.

Cut	Forage Stand	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	w/ Meadow Brome	6.0629	10.9592	43.6465	38.0491	1.1634	0.2158	1.3118	0.3254	0.3329
	Sainfoin Monoculture	6.4630	11.2518	44.7182	41.9120	1.2650	0.2640	1.6019	0.3526	0.3775
	P > F	*0.3085*	*0.6227*	*0.5412*	*0.3776*	*0.1526*	*0.0827*	*0.2866*	*0.2070*	*0.1466*
	SE	*0.3103*	*0.4269*	*2.5188*	*2.1099*	*0.0681*	*0.0168*	*0.1419*	*0.0257*	*0.0146*
Cut 2	w/ Meadow Brome	6.9231	14.6200	34.5020	33.3412	1.4064	0.1551	1.2897	0.4185	0.3329
	Sainfoin Monoculture	6.8289	14.3627	36.7986	33.6706	1.3684	0.1638	1.3489	0.4917	0.3725
	P > F	*0.7257*	*0.8736*	*0.3800*	*0.6218*	*0.7596*	*0.4485*	*0.7076*	*0.2949*	*0.2163*
	SE	*0.1906*	*0.8918*	*3.1768*	*2.7278*	*0.0940*	*0.0055*	*0.0975*	*0.0849*	*0.0174*

Appendix 8-8: Comparison of the proximate analysis of 2007 meadow brome grown in a monoculture and in mixture with both sainfoin and alfalfa. Estimate is made between meadow brome in alfalfa (A) and in sainfoin (S) mixtures.

Cut	Forage Stand	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	in Alfalfa Mixture	9.1482	8.9343	66.2843 a	43.8822	0.3685	0.2179	2.5907 a	0.1507	0.1507
	Monoculture	7.8164	5.4710	62.9684 b	41.9555	0.3449	0.1685	1.5868 b	0.1335	0.1335
	in Sainfoin Mixture	8.9996	8.1862	63.1040 b	42.0797	0.3843	0.2093	2.2702 b	0.1606	0.1606
	P > F	*0.1286*	*0.0107*	*0.0291*	*0.1407*	*0.7325*	*0.1974*	*0.0326*	*0.1320*	*0.5658*
	SE	*0.3730*	*0.4981*	*0.7009*	*0.5418*	*0.0331*	*0.0255*	*0.1791*	*0.0078*	*0.0239*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Cut 2	in Alfalfa Mixture	8.7400	10.9858	70.1918 a	43.2496 a	0.4620	0.1683 a	2.9036	0.2276	0.4397
	Monoculture	10.9499	8.3178	59.0535 b	39.3658 b	0.5594	0.1955	2.1880	0.2704	0.4082
	in Sainfoin Mixture	10.4764	9.3053	60.3801 b	38.6522 b	0.4821	0.2774 b	2.7122	0.3170	0.4057
	P > F	*0.4681*	*0.4993*	*<0.0001*	*0.0050*	*0.8105*	*0.1013*	*0.3336*	*0.4835*	*0.6432*
	SE	*1.0815*	*1.3885*	*0.5555*	*0.6057*	*0.1203*	*0.0433*	*0.2986*	*0.0485*	*0.0291*
	P>F A-S Estimate	*ns*	*ns*	*<0.0001*	*0.0024*	*ns*	*0.0454*	*ns*	*ns*	*ns*

Appendix 8-9: Proximate analysis means comparison between the five main-plot forage treatments in 2007, in two cuts. Estimates are made between the two legumes – alfalfa and sainfoin – and the two species mixtures (sainfoin and alfalfa with meadow brome).

Cut	Forage	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	Alfalfa	8.3248 a	15.5796 a	48.3578 bc	39.4727	1.5133 a	0.1958 b	2.0559 a	0.3257 a	0.4031
	Alfalfa + Meadow Brome	8.2028 a	14.3274 a	51.7008 b	39.6444	1.3111 ab	0.1983 b	1.9486 a	0.3235 a	0.4118
	Meadow Brome	7.8164 ab	5.4716 c	62.9684 a	41.9555	0.3449 d	0.1685 b	1.5868 b	0.1335 c	0.3914
	Sainfoin	6.4630 c	11.2518 b	44.7182 c	41.9120	1.2650 ab	0.2641 a	1.6019 b	0.3526 a	0.3775
	Sainfoin + Meadow Brome	7.3490 b	9.6159 b	53.4363 b	40.2920	0.7764 c	0.2095 b	1.7223 ab	0.2400 b	0.3654
	P > F	*0.0019*	*<0.0001*	*0.0002*	*0.6447*	*<0.0001*	*0.0102*	*0.0320*	*<0.0001*	*0.3297*
	SE	*0.2365*	*0.5789*	*1.8604*	*1.5830*	*0.0898*	*0.0143*	*0.1033*	*0.0193*	*0.0161*
	P>F A-S Estimate	*0.0003*	*0.0004*	*0.1943*	*0.4254*	*0.0840*	*0.0078*	*0.0115*	*0.3895*	*0.2959*
	P>F AMix-SMix Estimate	*0.0398*	*0.0002*	*0.5245*	*0.8947*	*0.0016*	*0.6091*	*0.1636*	*0.0168*	*0.0710*
Cut 2	Alfalfa	7.7464 c	16.4754 a	47.5784 b	38.2404	1.3358 a	0.1785 b	1.9172 a	0.3463	0.4532 a
	Alfalfa + Meadow Brome	7.7128 cd	17.0373 a	46.3337 b	36.1261	1.1383 a	0.1793 b	1.9490 a	0.3673	0.4583 a
	Meadow Brome	10.9499 a	8.3178 c	59.0535 a	39.3658	0.5594 b	0.1955 a	2.1880 a	0.2704	0.4082 ab
	Sainfoin	6.8289 d	14.3627 ab	36.7986 c	33.6706	1.3684 a	0.1638 b	1.3489 b	0.4917	0.3725 b
	Sainfoin + Meadow Brome	8.7237 b	12.0332 b	47.7032 b	35.7649	0.9704 a	0.2124 a	1.9619 a	0.3618	0.3816 b
	P > F	*<0.0001*	*<0.0001*	*0.0004*	*0.1411*	*0.0064*	*0.0025*	*0.0453*	*0.1057*	*0.0149*
	SE	*0.3051*	*0.7455*	*2.3894*	*1.8322*	*0.1314*	*0.0074*	*0.1897*	*0.0525*	*0.0219*
	P>F A-S Estimate	*0.0443*	*0.0813*	*0.0066*	*0.0563*	*0.8657*	*0.1453*	*0.0356*	*0.0674*	*0.0083*
	P>F AMix-SMix Estimate	*0.0292*	*0.0007*	*0.6843*	*0.8702*	*0.3914*	*0.0043*	*0.9580*	*0.9409*	*0.0110*

Appendix 8-10: Proximate analysis of 2008 alfalfa grown in a monoculture, means comparison between sub-plots treated with alfalfa manure and sainfoin manure at 44.8 t ha^{-1} and a non-manured control. Means estimate is made between the alfalfa (A) and sainfoin (S) manures.

Cut	Manure Treatment	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	Alfalfa Manure	9.4587	17.6478	43.9021	35.9677	1.5086 b	0.1902	2.8018 a	0.3176 b	0.4052
	Control (no manure)	9.4178	20.0298	42.1092	34.0180	1.9403 a	0.1873	2.0229 b	0.4045 a	0.3886
	Sainfoin Manure	9.6897	18.0581	43.6612	35.7113	1.5164 b	0.2141	2.7968 a	0.3332 b	0.3889
	P > F	*0.7332*	*0.1334*	*0.5011*	*0.5415*	*0.0062*	*0.2171*	*0.0059*	*0.0059*	*0.9579*
	SE	*0.2350*	*0.7062*	*1.1993*	*1.2715*	*0.0811*	*0.0121*	*0.1250*	*0.0299*	*0.0506*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*0.9377*	*ns*	*0.9777*	*0.4136*	*ns*
Cut 2	Alfalfa Manure	8.6963	20.7068	35.4371	29.4738	1.3800	0.1960	2.4314	0.3142	0.3510
	Control (no manure)	8.4038	19.9733	38.8886	31.6109	1.6037	0.1841	2.0345	0.3285	0.3285
	Sainfoin Manure	8.8165	20.0431	40.0265	32.4303	1.3936	0.1989	2.4243	0.3178	0.3178
	P > F	*0.3110*	*0.7731*	*0.3880*	*0.6592*	*0.2802*	*0.3352*	*0.1009*	*0.9411*	*0.1500*
	SE	*0.2500*	*0.7328*	*2.0314*	*2.2996*	*0.1030*	*0.0099*	*0.1543*	*0.0422*	*0.0263*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

Appendix 8-11: Proximate analysis of 2008 meadow brome grown in a monoculture, with means comparison between sub-plots treated with alfalfa manure and sainfoin manure at 44.8 t ha^{-1}, and a non-manured control. Means estimate is made between the alfalfa (A) and sainfoin (S) manures.

Cut	Manure Treatment	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	Alfalfa Manure	6.9638	6.7498	71.9410	43.9007	0.2683	0.1264 b	2.3991	0.1018	0.3420
	Control (no manure)	6.2834	5.8334	70.3647	42.8284	0.2653	0.1458 ab	2.0884	0.1013	0.3498
	Sainfoin Manure	6.6534	6.1978	71.2571	43.6562	0.2300	0.1690 a	2.1989	0.0967	0.3650
	P > F	*0.4363*	*0.1318*	*0.5739*	*0.3366*	*0.1534*	*0.0382*	*0.4532*	*0.5713*	*0.7894*
	SE	*0.3429*	*0.2334*	*0.8781*	*0.4345*	*0.0146*	*0.0124*	*0.1486*	*0.0071*	*0.0205*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*ns*	*0.0139*	*ns*	*ns*	*ns*
Cut 2	Alfalfa Manure	9.9125	10.8249	59.7200	37.8124	0.4854 a	0.1795	2.2600 a	0.2382	0.3531
	Control (no manure)	10.2968	9.8487	59.1461	37.8049	0.4897 a	0.1775	1.9199 b	0.2398	0.3901
	Sainfoin Manure	9.3778	10.3308	61.4006	38.7525	0.4310 b	0.1962	1.9495 b	0.2151	0.3789
	P > F	*0.2702*	*0.4823*	*0.0629*	*0.6415*	*0.0035*	*0.4353*	*0.0237*	*0.4894*	*0.4025*
	SE	*0.4325*	*0.8492*	*0.7568*	*0.9365*	*0.0312*	*0.0101*	*0.1343*	*0.0209*	*0.0250*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*0.0030*	*ns*	*0.0190*	*ns*	*ns*

Appendix 8-12: Proximate analysis of 2008 sainfoin grown in a monoculture, with means comparison between sub-plots treated with alfalfa manure and sainfoin manure at 44.8 t ha^{-1}, and a non-manured control. Means estimate is made between the alfalfa (A) and sainfoin (S) manures.

Cut	Manure Treatment	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	Alfalfa Manure	6.6265	13.5214	43.2876	38.4334	0.9354 ab	0.2440	1.8389	0.3233 b	0.3632
	Control (no manure)	6.1673	13.8739	43.9803	38.3258	1.0603 a	0.2392	1.4387	0.3493 a	0.3573
	Sainfoin Manure	6.4530	12.2712	44.6319	38.2203	0.8710 b	0.2384	1.8567	0.3059 b	0.3672
	P > F	*0.4501*	*0.1592*	*0.8834*	*0.9953*	*0.0422*	*0.8119*	*0.0616*	*0.0073*	*0.9347*
	SE	*0.2618*	*0.5752*	*1.7060*	*1.5516*	*0.0589*	*0.0111*	*0.1144*	*0.0218*	*0.0251*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*0.3049*	*ns*	*ns*	*0.0934*	*ns*
Cut 2	Alfalfa Manure	6.2242	15.2019	36.5878	32.7351	0.9947	0.1863	1.5536	0.4244	0.3429
	Control (no manure)	6.8136	16.2958	34.9249	31.2725	1.3200	0.1833	1.4067	0.4180	0.3734
	Sainfoin Manure	6.5586	15.3103	40.4847	36.4330	1.0723	0.1852	1.6589	0.3919	0.3435
	P > F	*0.4820*	*0.1359*	*0.2064*	*0.2066*	*0.2073*	*0.8808*	*0.1978*	*0.7465*	*0.4315*
	SE	*0.3308*	*0.9829*	*2.0428*	*1.9328*	*0.1108*	*0.0055*	*0.1345*	*0.0797*	*0.0204*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

Appendix 8-13: Proximate analysis of 2008 alfalfa component of an alfalfa + meadow brome mixture, with means comparison between sub-plots treated with alfalfa manure and sainfoin manure at 44.8 t ha^{-1}, and a non-manured control. Means estimate is made between the alfalfa (A) and sainfoin (S) manures.

Cut	Manure Treatment	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	Alfalfa Manure	9.5254	18.4258	41.3047	31.3835	1.6410	0.1700 b	2.4008	0.4807	0.3835
	Contol (no manure)	9.8303	18.3407	39.9064	30.9161	1.8691	0.1690 b	2.1046	0.5089	0.3648
	Sainfoin Manure	9.6435	18.0448	44.9648	33.4539	1.4043	0.1933 a	2.6982	0.4073	0.3989
	P > F	*0.5910*	*0.9027*	*0.1289*	*0.1146*	*0.1831*	*0.0040*	*0.1663*	*0.1246*	*0.5698*
	SE	*0.2833*	*0.7455*	*2.0014*	*1.0927*	*0.1677*	*0.0065*	*0.1645*	*0.0725*	*0.0307*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*ns*	*0.0030*	*ns*	*ns*	*ns*
Cut 2	Alfalfa Manure	8.1756	20.1362	37.9379	29.6783	1.3912	0.1845 a	2.0712	0.4130	0.4037
	Contol (no manure)	7.8276	18.8361	41.8660	32.6860	1.3751	0.1669 b	1.9374	0.3625	0.4195
	Sainfoin Manure	8.2764	19.1540	42.0128	33.5881	1.2382	0.1764 ab	2.2769	0.3641	0.3737
	P > F	*0.0888*	*0.1905*	*0.2005*	*0.2099*	*0.4828*	*0.0122*	*0.2665*	*0.5319*	*0.0567*
	SE	*0.1726*	*0.6670*	*2.7025*	*2.3882*	*0.1313*	*0.0039*	*0.1271*	*0.0800*	*0.0150*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*ns*	*0.0846*	*ns*	*ns*	*ns*

Appendix 8-14: Proximate analysis of the 2008 meadow brome component of an alfalfa + meadow brome mixture, with means comparison between sub-plots treated with alfalfa manure and sainfoin manure at 44.8 t ha^{-1}, and a non-manured control. Means estimate is made between the alfalfa (A) and sainfoin (S) manures.

Cut	Manure Treatment	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	Alfalfa Manure	7.2093	8.4588	67.4446	41.6037	0.3120	0.1845	2.6566	0.1203	0.3969
	Contol (no manure)	7.5606	9.1418	67.1160	40.1191	0.3219	0.1956	2.7107	0.1181	0.4234
	Sainfoin Manure	7.4027	8.8525	67.6555	40.9633	0.3093	0.1895	2.6154	0.1126	0.4063
	P > F	*0.7515*	*0.5143*	*0.8664*	*0.5143*	*0.7984*	*0.9010*	*0.8139*	*0.6906*	*0.5596*
	SE	*0.5867*	*1.3985*	*0.9358*	*0.9172*	*0.0367*	*0.0268*	*0.2786*	*0.0120*	*0.0229*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Cut 2	Alfalfa Manure	9.8231	15.0536	56.9595	37.3460	0.5397	0.2126	3.4507	0.2755	0.3993
	Contol (no manure)	10.1006	13.4962	62.3745	38.1613	0.5577	0.1965	3.2351	0.2625	0.4096
	Sainfoin Manure	9.6875	14.0867	61.3896	37.5361	0.4732	0.1945	3.4493	0.2335	0.4017
	P > F	*0.9399*	*0.6812*	*0.4145*	*0.9151*	*0.3241*	*0.6886*	*0.8842*	*0.3502*	*0.9612*
	SE	*0.7704*	*1.1804*	*2.9863*	*1.7580*	*0.0533*	*0.0141*	*0.3120*	*0.0346*	*0.0296*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

Appendix 8-15: Proximate analysis of the 2008 sainfoin component of an sainfoin + meadow brome mixture, with means comparison between sub-plots treated with alfalfa manure and sainfoin manure at 44.8 t ha^{-1}, and a non-manured control. Means estimate is made between the alfalfa (A) and sainfoin (S) manures.

Cut	Manure Treatment	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	Alfalfa Manure	6.5970	12.1143 b	43.5940	38.5471	0.9160	0.2163	1.8426	0.3245	0.3190
	Contol (no manure)	6.3044	12.7680	43.2654	37.8180	0.9440	0.2210	1.6919	0.3612	0.3678
	Sainfoin Manure	6.1269	14.1844 a	42.1213	36.8864	0.9797	0.2249	1.7433	0.3568	0.3278
	P > F	*0.5088*	*0.0595*	*0.7434*	*0.5861*	*0.5610*	*0.7507*	*0.5275*	*0.6053*	*0.2055*
	SE	*0.2556*	*0.4495*	*1.8348*	*2.0029*	*0.0505*	*0.0119*	*0.1029*	*0.0377*	*0.0276*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Cut 2	Alfalfa Manure	6.2657	15.1022	36.0044 ab	32.3677	1.1317	0.1644	1.5458 a	0.3523	0.3650
	Contol (no manure)	6.0603	15.5411	37.2406 a	34.0609	1.0797	0.1566	1.3706 ab	0.3928	0.3255
	Sainfoin Manure	6.1234	13.9736	32.2638 b	29.3240	1.2568	0.1461	1.1935 b	0.4241	0.3561
	P > F	*0.8127*	*0.3444*	*0.0413*	*0.0877*	*0.3756*	*0.5252*	*0.0287*	*0.3168*	*0.3914*
	SE	*0.2928*	*0.6730*	*2.0885*	*2.0252*	*0.0833*	*0.0113*	*0.1099*	*0.0649*	*0.0228*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*0.0102*	*ns*	*ns*

Appendix 8-16: Proximate analysis of the 2008 meadow brome component of an sainfoin + meadow brome mixture, with means comparison between sub-plots treated with alfalfa manure and sainfoin manure at 44.8 t ha^{-1}, and a non-manured control. Means estimate is made between the alfalfa (A) and sainfoin (S) manures.

Cut	Manure Treatment	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	Alfalfa Manure	6.6668	7.6422	69.7128 b	43.0509	0.2606	0.1790 a	2.5719 a	0.1060	0.4403
	Contol (no manure)	2.9423	7.3496	71.6197 a	43.8475	0.2455	0.1396 b	2.1292 b	0.1118	0.3877
	Sainfoin Manure	6.4847	7.5092	70.8023 a	43.8170	0.2654	0.1963 a	2.4036 ab	0.1028	0.4274
	P > F	*0.1224*	*0.4956*	*0.0042*	*0.4924*	*0.3239*	*0.0159*	*0.0366*	*0.1258*	*0.6750*
	SE	*0.2002*	*0.2500*	*0.7603*	*0.5830*	*0.0125*	*0.0124*	*0.0776*	*0.0100*	*0.0397*
	P>F A-S Estimate	*ns*	*ns*	*0.0191*	*ns*	*ns*	*0.2548*	*ns*	*ns*	*ns*
Cut 2	Alfalfa Manure	9.6846	12.2247	60.2199	38.3422	0.4535	0.2178	3.0311	0.2324	0.3790
	Contol (no manure)	8.7934	12.4967	62.1037	38.2995	0.5173	0.2365	2.6893	0.2252	0.4375
	Sainfoin Manure	8.8758	12.7793	60.1050	36.3596	0.4806	0.2313	2.7279	0.2419	0.4016
	P > F	*0.2208*	*0.8741*	*0.3920*	*0.0995*	*0.2220*	*0.8290*	*0.1849*	*0.7298*	*0.3483*
	SE	*0.3249*	*0.6923*	*1.3123*	*0.7614*	*0.0247*	*0.0228*	*0.1062*	*0.0287*	*0.0267*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

Appendix 8-17: Comparison of the proximate analysis of 2008 alfalfa grown in a monoculture and in mixture with meadow brome, each with 44.8 t ha^{-1} of alfalfa-derived solid beef manure applied the previous fall.

Cut	Forage Stand	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	w/ Meadow Brome	9.5294	18.4258	41.3047	31.3835	1.6410	0.1700 b	2.4008	0.4807	0.3855
	Alfalfa Monoculture	9.4587	17.6478	43.9021	35.9677	1.5087	0.1902 a	2.8018	0.3176	0.4053
	P > F	*0.7410*	*0.4038*	*0.2404*	*0.0737*	*0.3317*	*0.0382*	*0.0567*	*0.1063*	*0.7117*
	SE	*0.2503*	*0.5453*	*1.1400*	*1.1246*	*0.0833*	*0.0056*	*0.1213*	*0.0539*	*0.0624*
Cut 2	w/ Meadow Brome	8.1756	20.1362	37.9380	29.6783	1.3912	0.1845	2.0711 b	0.4130	0.4037
	Alfalfa Monoculture	8.6963	20.7068	35.4371	29.4738	1.3800	0.1961	2.4314 a	0.3142	0.3510
	P > F	*0.1758*	*0.6405*	*0.1799*	*0.9509*	*0.9275*	*0.2713*	*0.0026*	*0.3654*	*0.2187*
	SE	*0.2480*	*0.7222*	*2.6371*	*2.6141*	*0.0869*	*0.0045*	*0.1027*	*0.0797*	*0.0201*

Appendix 8-18: Comparison of the proximate analysis of 2008 alfalfa grown in a monoculture and in mixture with meadow brome with no manure applied (control treatment).

Cut	Forage Stand	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	w/ Meadow Brome	9.8303	18.3407	39.9064	30.9161	1.8691	0.1690	2.1047	0.5089	0.3648
	Alfalfa Monoculture	9.4178	20.0298	42.1092	34.0180	1.9403	0.1873	2.0229	0.4045	0.3886
	P > F	*0.4038*	*0.1136*	*0.1814*	*0.1256*	*0.8392*	*0.3758*	*0.8201*	*0.3171*	*0.3315*
	SE	*0.2454*	*0.7468*	*1.3635*	*1.1832*	*0.1985*	*0.0115*	*0.2037*	*0.0555*	*0.0159*
Cut 2	w/ Meadow Brome	7.8276 b	18.8361	41.8660	32.6860	1.3751	0.1664	1.9374	0.3625	0.4195
	Alfalfa Monoculture	8.4037 a	19.9733	38.8886	31.6109	1.6038	0.1841	2.0345	0.3285	0.4177
	P > F	*0.0203*	*0.4476*	*0.4635*	*0.6997*	*0.2070*	*0.3547*	*0.6544*	*0.4603*	*0.9260*
	SE	*0.1371*	*0.7122*	*1.8743*	*1.2991*	*0.1709*	*0.0097*	*0.2028*	*0.0316*	*0.0184*

Appendix 8-19: Comparison of the proximate analysis of 2008 alfalfa grown in a monoculture and in mixture with meadow brome, each with 44.8 t ha^{-1} of sainfoin-derived solid beef manure applied the previous fall.

Cut	Forage Stand	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	w/ Meadow Brome	9.6435	18.0448	44.9648	33.4539	1.4043	0.1933	2.6982	0.4073	0.3989
	Alfalfa Monoculture	9.6897	18.0581	43.6612	35.7113	1.5165	0.2141	2.7968	0.3332	0.3889
	P > F	*0.8767*	*0.9914*	*0.5989*	*0.2410*	*0.4417*	*0.3275*	*0.4076*	*0.4364*	*0.7776*
	SE	*0.2057*	*0.8525*	*2.2377*	*1.2455*	*0.0754*	*0.0108*	*0.0884*	*0.0569*	*0.0187*
Cut 2	w/ Meadow Brome	8.2764	19.1540	42.0128	33.5880	1.2382	0.1764	2.2769	0.3641	0.3737
	Alfalfa Monoculture	8.8165	20.0431	40.0265	32.4303	1.3936	0.1989	2.4243	0.3178	0.3771
	P > F	*0.2525*	*0.2918*	*0.1617*	*0.5163*	*0.3364*	*0.1780*	*0.2152*	*0.3925*	*0.9052*
	SE	*0.2059*	*0.6663*	*2.9040*	*2.8226*	*0.0706*	*0.0074*	*0.0909*	*0.0701*	*0.0225*

Appendix 8-20: Comparison of the proximate analysis of 2008 sainfoin grown in a monoculture and in mixture with meadow brome, each with 44.8 t ha^{-1} of alfalfa-derived solid beef manure applied the previous fall.

Cut	Forage Stand	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	w/ Meadow Brome	6.5970	12.1150	43.5939	38.5471	0.9160	0.2163	1.8426	0.3245	0.3190
	Sainfoin Monoculture	6.6265	13.5214	43.2876	38.4334	0.9354	0.2440	1.8389	0.3234	0.3632
	P > F	*0.9185*	*0.2078*	*0.8344*	*0.9182*	*0.8378*	*0.2538*	*0.9795*	*0.9657*	*0.4403*
	SE	*0.2617*	*0.7368*	*1.3895*	*1.3213*	*0.0624*	*0.0113*	*0.1121*	*0.0209*	*0.0310*
Cut 2	w/ Meadow Brome	6.2657	15.1022	36.0044	32.3677	1.1317	0.1644	1.5458	0.3523	0.3650
	Sainfoin Monoculture	6.2242	15.2019	36.5878	32.7351	0.9947	0.1862	1.5536	0.4243	0.3429
	P > F	*0.9099*	*0.8767*	*0.6314*	*0.7731*	*0.4132*	*0.1096*	*0.9475*	*0.0839*	*0.4621*
	SE	*0.3238*	*0.5101*	*1.0737*	*0.8715*	*0.1037*	*0.0107*	*0.0962*	*0.0528*	*0.0308*

Appendix 8-21: Comparison of the proximate analysis of 2008 sainfoin grown in a monoculture and in mixture with meadow brome with no manure applied (control treatment).

Cut	Forage Stand	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	w/ Meadow Brome	6.3044	12.7680	43.2654	37.8180	0.9440	0.2210	1.6919	0.3612	0.3678
	Sainfoin Monoculture	6.1673	13.8739	43.9803	38.3258	1.0603	0.2392	1.4386	0.3493	0.3573
	P > F	*0.6334*	*0.0676*	*0.2597*	*0.3708*	*0.1136*	*0.4107*	*0.1348*	*0.7314*	*0.8056*
	SE	*0.2277*	*0.2680*	*1.4796*	*1.6288*	*0.0424*	*0.0115*	*0.1159*	*0.0434*	*0.0228*
Cut 2	w/ Meadow Brome	6.0603	15.5411	37.2406	34.0609	1.0797	0.1566	1.3706	0.3928	0.3255 b
	Sainfoin Monoculture	6.8136	16.2958	34.9249	31.2725	1.3200	0.1833	1.4067	0.4180	0.3733 a
	P > F	*0.3684*	*0.1485*	*0.5481*	*0.4135*	*0.3083*	*0.1498*	*0.8348*	*0.5230*	*0.0028*
	SE	*0.3663*	*0.3365*	*2.5439*	*2.5319*	*0.1079*	*0.0084*	*0.1592*	*0.0939*	*0.0093*

Appendix 8-22: Comparison of the proximate analysis of 2008 sainfoin grown in a monoculture and in mixture with meadow brome, each with 44.8 t ha^{-1} of sainfoin-derived solid beef manure applied the previous fall.

Cut	Forage Stand	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	w/ Meadow Brome	6.1269	14.1845	42.1213	36.8864	0.9797	0.2249	1.7433	0.3568 a	0.3278
	Sainfoin Monoculture	6.4530	12.2706	44.6319	38.2203	0.8710	0.2384	1.8567	0.3059 b	0.3671
	P > F	*0.5829*	*0.0537*	*0.5677*	*0.7020*	*0.1516*	*0.4445*	*0.3669*	*0.0259*	*0.1255*
	SE	*0.2836*	*0.4307*	*2.3012*	*2.2869*	*0.0576*	*0.0116*	*0.0975*	*0.0227*	*0.0246*
Cut 2	w/ Meadow Brome	6.1234	13.9736	32.2638 b	29.3241 b	1.2568	0.1460 b	1.1935 b	0.4241	0.3561
	Sainfoin Monoculture	6.5586	15.3103	40.4847 a	36.4330 a	1.0723	0.1852 a	1.6589 a	0.3919	0.3435
	P > F	*0.0587*	*0.3555*	*0.0123*	*0.0273*	*0.0892*	*0.0049*	*0.0042*	*0.2779*	*0.5664*
	SE	*0.2319*	*0.6763*	*2.2755*	*2.1414*	*0.0802*	*0.0071*	*0.1033*	*0.0650*	*0.0194*

Appendix 8-23: Comparison of the proximate analysis of 2008 meadow brome grown in a monoculture and in mixtures with both alfalfa and sainfoin, each with 44.8 t ha^{-1} of alfalfa-derived solid beef manure applied the previous fall.

Cut	Forage Stand	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	in Alfalfa Mixture	7.2093	8.4588	67.4446 b	41.6037 b	0.3120	0.1845	2.6566	0.1203	0.3969
	Monoculture	6.9638	6.7498	71.9410 a	43.9007 a	0.2683	0.1264	2.3991	0.1018	0.342
	in Sainfoin Mixture	6.6668	7.6422	69.7128 a	43.0509 a	0.2606	0.1790	2.5719	0.1060	0.4403
	P > F	*0.5765*	*0.1672*	*0.0079*	*0.0158*	*0.3528*	*0.0634*	*0.1878*	*0.4209*	0.3087
	SE	*0.3736*	*0.6510*	*0.6774*	*0.4377*	*0.0231*	*0.0145*	*0.1550*	*0.0106*	0.043
	P>F A-S Estimate	*ns*	*ns*	*0.0479*	*0.0388*	*ns*	*ns*	*ns*	*ns*	ns
Cut 2	in Alfalfa Mixture	9.7272	15.0536 a	56.9595	37.3460	0.5397	0.2126	3.4507 a	0.2756	0.3993
	Monoculture	9.9125	10.8242 b	59.7200	37.8124	0.4854	0.1795	2.2600 b	0.2382	0.3531
	in Sainfoin Mixture	9.6846	12.2247 ab	60.2199	38.3421	0.4535	0.2178	3.0310 a	0.2324	0.379
	P > F	*0.9102*	*0.0348*	*0.7314*	*0.8952*	*0.2904*	*0.1921*	*0.0023*	*0.6292*	0.428
	SE	*0.3452*	*0.7302*	*2.8761*	*1.6369*	*0.0332*	*0.0144*	*0.1671*	*0.0296*	0.0228
	P>F A-S Estimate	*ns*	*0.0602*	*ns*	*ns*	*ns*	*ns*	*0.0724*	*ns*	ns

Appendix 8-24: Comparison of the proximate analysis of 2008 meadow brome grown in a monoculture and in mixtures with both alfalfa and sainfoin, with no manure applied (control treatment).

Cut	Forage Stand	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	in Alfalfa Mixture	7.5606 a	9.1424	67.1159 b	40.1191 c	0.3219	0.1956	2.7107	0.1182	0.4234 a
	Monoculture	6.2834	5.8340	70.3647 b	42.8284 b	0.2653	0.1459	2.0884	0.1013	0.3498 b
	in Sainfoin Mixture	5.9423 b	7.3502	71.6197 a	43.8475 a	0.2455	0.1396	2.1293	0.1118	0.3877 b
	P > F	*0.0805*	*0.1071*	*0.0399*	*0.0329*	*0.2174*	*0.1695*	*0.1698*	*0.5653*	0.0492
	SE	*0.4090*	*0.8399*	*1.0609*	*0.7634*	*0.0266*	*0.0173*	*0.1988*	*0.0105*	0.0141
	P>F A-S Estimate	*0.0372*	*ns*	*0.0165*	*0.0136*	*ns*	*ns*	*ns*	*ns*	0.1698
Cut 2	in Alfalfa Mixture	9.7617	13.4962 a	63.3745	38.1613	0.5577	0.1965	3.2351 a	0.2625	0.4096
	Monoculture	10.2968	9.8487 b	59.1461	37.8049	0.4897	0.1775	1.9199 b	0.2398	0.3901
	in Sainfoin Mixture	8.7934	12.4973 a	62.1037	38.2995	0.5172	0.2365	2.6893 a	0.2552	0.4375
	P > F	*0.1630*	*0.0171*	*0.0767*	*0.8068*	*0.5829*	*0.1042*	*0.0168*	*0.8916*	0.6361
	SE	*0.4734*	*0.8978*	*1.1337*	*0.8820*	*0.0415*	*0.0187*	*0.2042*	*0.0311*	0.03
	P>F A-S Estimate	*ns*	*0.3129*	*ns*	*ns*	*ns*	*ns*	*0.1353*	*ns*	ns

Appendix 8-25: Comparison of the proximate analysis of 2008 meadow brome grown in a monoculture and in mixtures with both alfalfa and sainfoin, each with 44.8 t ha^{-1} of sainfoin-derived solid beef manure applied the previous fall.

Cut	Forage Stand	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	in Alfalfa Mixture	7.4028	8.8531	67.6555 b	40.9633 b	0.3093	0.1895	2.6154	0.1126	0.4063
	Monoculture	6.6534	6.1978	71.2571 a	43.6562 a	0.2300	0.1690	2.1989	0.0967	0.3651
	in Sainfoin Mixture	6.4847	7.5098	70.8023 a	43.8170 a	0.2654	0.1963	2.4036	0.1028	0.4274
	P > F	*0.2894*	*0.1532*	*0.0398*	*0.0317*	*0.0926*	*0.6398*	*0.2340*	*0.3205*	0.0991
	SE	*0.4416*	*0.9716*	*0.8003*	*0.7718*	*0.0218*	*0.0228*	*0.2054*	*0.0087*	0.0217
	P>F A-S Estimate	*ns*	*ns*	*0.0342*	*0.0185*	*ns*	*ns*	*ns*	*ns*	ns
Cut 2	in Alfalfa Mixture	9.6875	14.0867	61.3896	37.5362	0.4732	0.1946	3.4493 a	0.2336	0.4017
	Monoculture	9.3778	10.3308	61.4006	38.7525	0.4310	0.1962	1.9495 b	0.2151	0.3789
	in Sainfoin Mixture	8.8758	12.7793	60.1050	36.3596	0.4806	0.2313	2.7279 b	0.2419	0.4016
	P > F	*0.6884*	*0.1826*	*0.7404*	*0.3567*	*0.6815*	*0.1791*	*0.0152*	*0.7753*	0.8273
	SE	*0.6019*	*1.0959*	*1.2868*	*1.0439*	*0.0400*	*0.0161*	*0.2388*	*0.0249*	0.0282
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*0.0858*	*ns*	ns

Appendix 8-26: Year comparison of the proximate analyses of alfalfa grown in monoculture, receiving no manure (control treatment) the previous fall.

Cut	Year	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	2007	8.3248 b	15.5796 b	48.3578 a	39.4727 a	1.5133 b	0.1958	2.0558	0.3257 b	0.4031
	2008	9.4178 a	20.0298 a	42.1092 b	34.0180 b	1.9403 a	0.1873	2.0229	0.4045 a	0.3886
	P > F	*0.0235*	*0.0170*	*0.0325*	*0.0498*	*0.0247*	*0.3916*	*0.7937*	*0.0290*	*0.6400*
	SE	*0.1573*	*0.8080*	*1.6487*	*1.6135*	*0.1004*	*0.0148*	*0.1343*	*0.0319*	*0.0168*
Cut 2	2007	7.7464	16.4754 b	47.5784 a	38.2404 a	1.3358 b	0.1785	1.9172	0.3463	0.4532
	2008	8.4038	19.9733 a	38.8886 b	31.6109 b	1.6037 a	0.1841	2.0345	0.3285	0.4177
	P > F	*0.0830*	*0.0264*	*0.0114*	*0.0183*	*0.0069*	*0.5431*	*0.4225*	*0.5197*	*0.3670*
	SE	*0.2228*	*0.6584*	*1.1308*	*0.9846*	*0.1498*	*0.0100*	*0.2704*	*0.0156*	*0.0232*

Appendix 8-27: Year comparison of the proximate analyses of meadow brome grown in monoculture, receiving no manure (control treatment) the previous fall.

Cut	Year	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	2007	7.8164	5.4716	62.9684 b	41.9555	0.3449	0.1685	1.5868 b	0.1335	0.3914
	2008	6.2834	5.8334	70.3647 a	42.8284	0.2653	0.1458	2.0884 a	0.1013	0.3498
	P > F	*0.0766*	*0.5824*	*0.0074*	*0.2764*	*0.0730*	*0.0956*	*0.0408*	*0.1084*	*0.1046*
	SE	*0.3245*	*0.3498*	*1.0817*	*0.4326*	*0.0328*	*0.0064*	*0.1013*	*0.0084*	*0.0104*
Cut 2	2007	10.9499	8.3178	59.0535	39.3658	0.5594	0.1955	2.1880	0.2704	0.4082
	2008	10.2968	9.8487	59.1461	37.8049	0.4897	0.1775	1.9199	0.2398	0.3901
	P > F	*0.0606*	*0.2454*	*0.9352*	*0.0905*	*0.6547*	*0.4073*	*0.4507*	*0.3129*	*0.7475*
	SE	*0.5415*	*0.7767*	*0.6454*	*0.8001*	*0.1172*	*0.0115*	*0.1657*	*0.0261*	*0.0312*

Appendix 8-28: Year comparison between the proximate analyses of sainfoin grown in monoculture, receiving no manure (control treatment) the previous fall.

Cut	Year	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	2007	6.4630	11.2518 b	44.7182	41.9120	1.2650	0.2641	1.6018	0.3526	0.3775
	2008	6.1673	13.8732 a	43.9803	38.3258	1.0603	0.2392	1.4386	0.3493	0.3573
	P > F	*0.2000*	*0.0008*	*0.6546*	*0.3810*	*0.0704*	*0.4014*	*0.2103*	*0.8423*	*0.5107*
	SE	*0.3058*	*0.3169*	*2.4154*	*2.1275*	*0.0733*	*0.0165*	*0.1475*	*0.0246*	*0.0216*
Cut 2	2007	6.8289	14.3627	36.7986	33.6706	1.3684	0.1638	1.3489	0.4917	0.3725
	2008	6.8136	16.2958	34.9249	31.2725	1.3200	0.1833	1.4067	0.4180	0.3734
	P > F	*0.9706*	*0.2080*	*0.6505*	*0.5577*	*0.7697*	*0.0818*	*0.6450*	*0.1827*	*0.9026*
	SE	*0.2216*	*0.8738*	*3.5864*	*3.3578*	*0.1043*	*0.0041*	*0.1473*	*0.1057*	*0.0097*

Appendix 8-29: Year comparison of the proximate analyses of alfalfa + meadow brome, receiving no manure (control treatment) the previous fall. Values for the combined forages are calculated mathematically based upon proximate analyses of the component species and their % DM species composition.

Cut	Year	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	2007	8.2028	14.3274	51.7008	39.6444 a	1.3111	0.1983	1.9486	0.3235	0.4118
	2008	9.1177	15.4027	47.7511	33.8422 b	1.4470	0.1716	2.1842	0.3848	0.3855
	P > F	*0.0917*	*0.1391*	*0.1364*	*0.0072*	*0.4907*	*0.0853*	*0.2694*	*0.2467*	*0.2231*
	SE	*0.2843*	*0.7754*	*2.0876*	*1.0667*	*0.2143*	*0.0095*	*0.1045*	*0.0234*	*0.0138*
Cut 2	2007	7.7128	17.0373	46.3337	36.1261	1.1383	0.1793	1.9490	0.3673	0.4583
	2008	8.0060	17.8852	44.6717	33.4745	1.2669	0.1712	2.0962	0.3482	0.4139
	P > F	*0.4984*	*0.3145*	*0.4362*	*0.2514*	*0.5798*	*0.5705*	*0.5612*	*0.5819*	*0.3232*
	SE	*0.2119*	*0.5184*	*2.4519*	*1.3786*	*0.1407*	*0.0067*	*0.1675*	*0.0342*	*0.0213*

Appendix 8-30: Year comparison of the proximate analyses of sainfoin + meadow brome, receiving no manure (control treatment) the previous fall. Values for the combined forages are calculated mathematically based upon proximate analyses of the component species and their % DM species composition.

Cut	Year	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	2007	7.3490 a	9.6159	53.4363	40.2920	0.7764 a	0.2095	1.7223 b	0.2400	0.3654
	2008	6.0056 b	9.2488	61.8580	41.7388	0.4876 b	0.1688	1.9739 a	0.1971	0.3806
	P > F	0.0157	0.6157	0.0517	0.2768	0.0154	0.0832	0.0205	0.1712	0.6066
	SE	*0.2013*	*0.5360*	*1.9970*	*0.9181*	*0.0724*	*0.0154*	*0.1032*	*0.0195*	*0.0154*
Cut 2	2007	8.7237 a	12.0332 b	47.7032	35.7649	0.9704	0.2124	1.9619	0.3618	0.3816
	2008	7.1160 b	14.6253 a	45.7810	35.6166	0.8960	0.1806	1.8564	0.3361	0.3691
	P > F	0.0122	0.0263	0.5491	0.8964	0.6817	0.1396	0.6644	0.4953	0.6308
	SE	*0.2764*	*0.6903*	*2.0392*	*0.7898*	*0.1417*	*0.0096*	*0.1303*	*0.0406*	*0.0163*

Appendix 8-31: Comparison of the proximate analysis of alfalfa + meadow brome in 2008 between manure treatments and a manure-free control. Means estimate is made between alfalfa (A) and sainfoin (S) manure treatments.

Cut	Manure Treatment	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	Alfalfa Manure	8.7032	14.3893	51.3356 a	35.5254	1.1930 a	0.1754	2.4271	0.3231 a	0.3967
	Contol (no manure)	9.1177	15.4034	47.7511 b	33.8422	1.4470 a	0.1716	2.1842	0.3848 a	0.3855
	Sainfoin Manure	8.5965	13.6791	54.5793 a	36.6450	0.9580 b	0.1875	2.5889	0.2664 b	0.4052
	P > F	*0.2345*	*0.1421*	*0.0180*	*0.1022*	*0.0472*	*0.3752*	*0.2544*	*0.0463*	*0.7127*
	SE	*0.4479*	*1.2023*	*2.7750*	*1.0808*	*0.2218*	*0.0100*	*0.1307*	*0.2123*	*0.0165*
	P>F A-S Estimate	*ns*	*ns*	*0.0990*	*ns*	*ns*	*ns*	*ns*	*0.1683*	*ns*
Cut 2	Alfalfa Manure	8.0157	19.1354	42.1396	31.1670	1.2151	0.1921 a	2.3709	0.3866	0.4015 a
	Contol (no manure)	8.0060	17.8852	44.6717	33.4744	1.2669	0.1712 b	2.0961	0.3482	0.4139 b
	Sainfoin Manure	8.5909	18.1033	46.0387	34.5942	1.0952	0.1811 b	2.4852	0.3292	0.3738 c
	P > F	*0.4886*	*0.3696*	*0.5418*	*0.2911*	*0.5573*	*0.0180*	*0.3107*	*0.4256*	*0.0145*
	SE	*0.3783*	*0.7268*	*2.7630*	*2.1542*	*0.1351*	*0.0046*	*0.1493*	*0.0672*	*0.0086*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*ns*	*0.0740*	*ns*	*ns*	*0.0271*

Appendix 8-32: Comparison of the proximate analysis of sainfoin + meadow brome in 2008 between manure treatments and a manure-free control. Means estimate is made between alfalfa (A) and sainfoin (S) manure treatments.

Cut	Manure Treatment	Ash	Crude Protein	NDF	ADF	Calcium	Phosphorous	Potassium	Magnesium	Sodium
Cut 1	Alfalfa Manure	6.5961	9.0872	61.9645	41.7218	0.4683	0.1887	2.3431	0.1686	0.4186
	Contol (no manure)	6.0056	9.2482	61.8580	41.7388	0.4876	0.1688	1.9739	0.1970	0.3807
	Sainfoin Manure	6.4127	9.6325	63.1713	42.2533	0.4813	0.2060	2.2060	0.1715	0.3970
	P > F	*0.1462*	*0.5593*	*0.6278*	*0.8163*	*0.8748*	*0.0870*	*0.1154*	*0.5315*	*0.7721*
	SE	*0.1521*	*0.4857*	*2.5506*	*0.7865*	*0.0771*	*0.0148*	*0.1241*	*0.0172*	*0.0333*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Cut 2	Alfalfa Manure	8.4375	13.4430	51.0936	36.0587	0.7089	0.1981	2.4929 a	0.2743	0.3679
	Contol (no manure)	7.1160	14.6253	45.7810	35.6158	0.8960	0.1806	1.8564 b	0.3361	0.3691
	Sainfoin Manure	7.8347	13.7988	47.6970	34.2122	0.8791	0.1832	2.0305 ab	0.3064	0.3968
	P > F	*0.1467*	*0.1418*	*0.2804*	*0.1980*	*0.3300*	*0.4817*	*0.0483*	*0.2827*	*0.6223*
	SE	*0.3941*	*0.4495*	*2.2892*	*0.9555*	*0.1201*	*0.0104*	*0.1584*	*0.0355*	*0.0245*
	P>F A-S Estimate	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*0.0633*	*ns*	*ns*

Appendix 8-33: Plot plan of the Carman research site, including main plot forage treatments and sub-plot manure treatments.

==> North

Rep 4	Sainfoin	Alfalfa	Alfalfa	Alfalfa		Alfalfa		Alfalfa	Alfalfa
	Alfalfa		Sainfoin		Alfalfa		Sainfoin	Sainfoin	Sainfoin
		Sainfoin		Sainfoin	Sainfoin	Sainfoin	Alfalfa		
	6	**4**	**2**	**9**	**5**	**1**	**3**	**7**	**8**
Rep 3		Alfalfa	Sainfoin		Alfalfa	Sainfoin			Sainfoin
	Sainfoin			Alfalfa	Sainfoin	Alfalfa	Alfalfa	Sainfoin	Alfalfa
	Alfalfa	Sainfoin	Alfalfa	Sainfoin			Sainfoin	Alfalfa	
	2	**3**	**8**	**4**	**7**	**6**	**5**	**1**	**9**
Rep 2	Sainfoin		Alfalfa	Sainfoin	Alfalfa	Alfalfa	Alfalfa	Sainfoin	Sainfoin
	Alfalfa	Sainfoin	Sainfoin	Alfalfa	Sainfoin		Sainfoin		Alfalfa
		Alfalfa				Sainfoin		Alfalfa	
	5	**9**	**6**	**1**	**3**	**8**	**7**	**2**	**4**
Rep 1	Alfalfa	Sainfoin	Sainfoin	Alfalfa			Sainfoin	Alfalfa	Sainfoin
		Alfalfa	Alfalfa	Sainfoin	Sainfoin	Alfalfa	Alfalfa	Sainfoin	Alfalfa
	Sainfoin				Alfalfa	Sainfoin			
	1	**7**	**4**	**8**	**9**	**2**	**5**	**6**	**3**

Treatment Key					
	1	Alfalfa	**6**	Alfalfa + Meadow Brome	
	2	Birdsfoot Trefoil	**7**	Birdsfoot Trefoil + Meadow Brome	Sainfoin - Sanfoin Hay Manure App. @ 44.8 t/ha.
	3	Sainfoin	**8**	Sainfoin + Meadow Brome	Alfalfa - Alfalfa Hay Manure App. @ 44.8 t/ha.
	4	Purple Prairie Clover	**9**	Purple Prairie Clover + Meadow Brome	- No Manure (Control)
	5	Meadow Brome			