Sainfoin tannins and their impact on protein degradation during silage and rumen fermentation and testing of novel techniques

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Cover: Blooming sainfoin field and bumble bee
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Abstract

The legume species sainfoin *(Onobrychis viciifolia)* has been shown to contain tannins of particularly beneficial composition for ruminant nutrition. This thesis describes effects of condensed tannins on protein degradation in general and in sainfoin specifically. Five studies are presented that investigate 1) effects of different ensiling treatments on sainfoin, 2) *in vitro* rumen protein degradation of sainfoin related to tannin content, 3) effects of molecular tannin structure on binding of tannins to proteins 4) modifications of the inoculum in an *in vitro* protein degradation assay.

Results showed that sainfoin had promising ensiling characteristics using different levels of commercial silage acidifiers at dry matter levels ranging from 200 to 600 g/kg (Papers I and II). Buffer soluble nitrogen (BSN) content in silage was as low as 250 g/kg nitrogen when the protein sparing tannin effect was not inhibited by polyethylene glycol. With the addition of polyethylene glycol, BSN levels more than doubled. This protein sparing effect is based on the formation of insoluble tannin-protein complexes that cannot be digested by bacteria. However, these complexes may be unstable at low pH. Paper II showed that natural formation of acids during ensiling and treatment with acidic silage additives, resulting in pH as low as 3.67, did not influence the protein sparing effect of sainfoin tannins.

In vitro analysis (Paper IV) revealed that protein degradation was not correlated to tannin content according to the HCl/butanol or radial diffusion assays. Structural tannin characteristics such as degree of polymerization, ratio of cis to trans binding or ratio of procyanidins to prodelphinidins could not explain differences in protein precipitation (Paper V). Further, a promising *in vitro* protein degradation assay was successfully improved by defaunation of rumen fluid (Paper III).

In conclusion, sainfoin tannins showed potential to increase protein utilization by ruminants. It was however also shown that assays employed to measure tannin content were not able to predict nutritional responses probably caused by not yet analyzed factors such as tannin linkage patterns. Future research has to address molecular structure-dependant binding of tannins to proteins before further nutritional research on tannin specific effects on proteins are performed.

Keywords: ammonia, breakdown, bypass protein, condensed tannins, degree of polymerization, Esparsette, fermentation, gas production, *in vitro*, legume, rumen escape protein, sustainability

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Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Lorenz, M. M., Eriksson, T., Udén, P., (2010). Effect of wilting, silage additive, PEG treatment and tannin content on the distribution of N between different fractions after ensiling of three different sainfoin (*Onobrychis viciifolia*) varieties. Grass and Forage Science 65, 175-184.

II Lorenz, M. M., Udén, P. (2011). Influence of formic acid and dry matter on protein degradation in the tanniniferous legume sainfoin. Animal Feed Science and Technology 164, 217-224.

III Lorenz, M. M., Karlsson, L., Hetta, M., Udén, P. (2011). Recycling of microbial N and the estimation of protein degradation by *in vitro* gas production. (Short communication, In press, Animal Feed Science and Technology, DOI. 10.1016/j.anifeedsci.2011.07.012).

IV Lorenz, M. M., Udén, P. A correlation study between *in vitro* protein degradation and a wide range of tannin and protein contents of different sainfoin (*Onobrychis viciifolia*) varieties. (Under review, Animal Feed Science and Technology).

V Lorenz, M.M., Alkhafadji, L., Nilsson, S., Udén P. Condensed tannin size and structure dependant binding to proteins *in vitro*. (Short communication, Manuscript).

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Abbreviations

AA	α-amino acid
BSA	Bovine serum albumin
BSN	Buffer soluble nitrogen
CT	Condensed tannins
DM	Dry matter
$FUD_{(i)}$	Fraction undegraded protein at incubation hour i
IIV	Inhibited *in vitro*
IVDP	*In vitro* degradability of crude protein
NH_3	Ammonia
N	Nitrogen
PEG	Polyethylene glycol
RF	Ruminal fluid
Rubisco	Ribulose-1,5-bisphosphate carboxylase oxygenase

1 Introduction

European grasslands have been changing during the last 70 years. Driving forces were the availability of inexpensive energy and a political framework that supported production. Overproduction and a high burden on the environment were some of the consequences. New regulations and increased awareness towards ecological issues in combination with increasing costs, has pushed the development towards so called “sustainable” production systems (European Council, 2003). The project “Re-invention of sainfoin” within the EU research program “Healthyhay” (EU, FP6-2005-Mobility-1, February 14, 2006) is one such approach to characterize and scientifically test a formerly rife forage legume.

The legume sainfoin (*Onobrychis viciifolia*) was grown intensively since it was introduced from Asia to Europe in the sixteenth century. Most legumes grow in symbiosis with microbes that can utilize nitrogen (N) from the atmosphere. Legumes are, therefore, generally high in protein. Sainfoin combines benefits of high protein content with benefits that come with condensed tannins (CT). Condensed tannins are found in many plants as secondary metabolites and some may yield beneficial, others detrimental effects in animal nutrition. Sainfoin tannins have been reported to be beneficial for ruminant nutrition (Caygill & Mueller-Harvey, 1999; Karnezos *et al.*, 1994).

The rumen contains bacterial enzymes which are able to degrade most plant material and, thereby, provide the host with energy and high quality protein. However, protein supply might not be optimal if demand is high or diets are not well balanced. High urinary N output into the environment is an implication of sub-optimal protein utilization. Tannin-protein binding can improve protein utilization by protecting proteins from bacterial fermentation. These tannin-protein complexes are not digestible by bacterial enzymes and can pass undigested through the rumen. The bound proteins can then be dissociated at the low pH conditions of the abomasum (Oh & Hoff, 1987) to be

digested by gastric enzymes into amino acids (AA) which are absorbed from the intestine into the blood stream (Thomson *et al.*, 1971).

Tannins comprise a group of polymers of enormous molecular variation. Therefore it is crucial to screen tannin containing plants, such as sainfoin, to gain knowledge about their beneficial effect on the efficiency of protein utilization and, hence, reduction in N effluents from ruminants. Both effects may minimize feed costs and environmental impact.

Feeding regimes for dairy animals have to provide adequate protein quality and quantity for high producing dairy cows to meet the present demand for dairy products. Results are often high energy consumption, eutrophication, air pollution, soil erosion and monocultures. The challenge for sustainable agriculture is to meet these concerns.

2 Background

2.1 Proteins

Berzelius (1749-1848) and Mulder (1802-1880) were the first to understand the significance of a molecule that they described as:

"… protein from πρωτᾶος, primaries, which is combined with sulfur and phosphorus in egg white and fibrin… This is the foodstuff of the whole animal kingdom and is probably formed only by plants" (Jorpes, 1970; Vickery, 1950).

Nowadays, we know that protein biosynthesis is not restricted solely to plants. In fact, the plant protein biomass is far surpassed by maritime prokaryotic protein. The large numbers of different proteins are formed by a combination of 21 proteinogenic AA´s. Apart from carbon, nitrogen, oxygen and hydrogen, some AA´s also contain sulfur.

The AA sequence determines protein structure which can be categorized in for example solubility in various solvents, binding to other molecules or by their shape into fibrous or globular. These characteristics are important to consider as they affect the nutritive value, storage, transport, immunological and catalytic properties or structural compounds. All aspects of biological life involve the actions of proteins and every species has its own set of proteins that serve specific needs.

2.1.1 Proteins in ruminant nutrition

The rumen is one of four stomachs of a ruminant. It is the habitat of a large variety of microorganisms. Microbes live in symbiosis with the host animal and ferment fiber which is indigestible by mammalian enzymes. By doing so, they provide energy to the animal in form of short chained fatty acids mainly as acetate, propionate and butyrate. Bacteria satisfy their protein needs by metabolizing mainly dietary protein. They are also able to synthesize protein from simple N compounds such as salivary urea, ammonia (NH_3) and other

forms of non-protein N, given that sufficient energy for this process can be derived from the diet. Microbial and feed proteins account for the majority of protein supply to the animal. Intestinal enzymes degrade the incoming protein to peptides and AA´s in the abomasums with additional degradation also occurring in the small intestine. The AA´s can then be absorbed in the duodenum by active transport into the blood stream. These AA´s are either metabolized as energy substrate to carbon dioxide (CO_2) and NH_3, or used for tissue synthesis or milk, hair, etc.

Synthesis of microbial protein can improve protein quality. The AA composition of bacterial protein, although variable (Clark *et al.*, 1992), is considered to be of higher quality than most feed proteins for milk production (NRC, 2001). Microbial protein presents over 50% of metabolizable protein for basic diets (White & Ashes, 1999) but at high protein demands, dietary protein becomes increasingly important (Clark *et al.*, 1992). However, if protein content in the diet is high, degradation and outflow of urinary N increases. This results in a high metabolic NH_3 load for the liver. Increased production costs and environmental problems are the consequences (Huhtanen *et al.*, 2006). Strategies to improve protein utilization include diet optimization, balancing dietary AA composition and increasing rumen escape protein (Huhtanen & Hristov, 2009; NRC, 2001; Chalupa & Sniffen, 1996).

2.1.2 Protein feed for ruminants

The main source of protein for ruminant nutrition has traditionally been grasses and forage legumes. Legumes live in mutuality with Rhizobia. These bacteria are capable of reducing atmospheric N into NH_3, which they provide to the plant in exchange for energy. Hence, legumes generally have higher protein contents than non-leguminous plants in N deficient soils. In spite of their high protein levels, many forage legume diets may not be sufficient to meet the protein requirements of high-yielding dairy cows.

Within the last decades, the number of cows has decreased in the EU while, at the same time, milk production per cow has increased (Boschma *et al.*, 1999). High producing dairy cows need both high quality protein and high quantities of protein to produce an estimated 133 million tons of milk per year in the EU (Eurostat, 2010). Protein rich feed supplements such as soybean, rapeseed, or sunflower meal from oil production or distillers grain are, therefore, used to supplement diets. Soybean production in South America is a classic example of a successful development and exploitation of legume seed proteins (Figure 1).

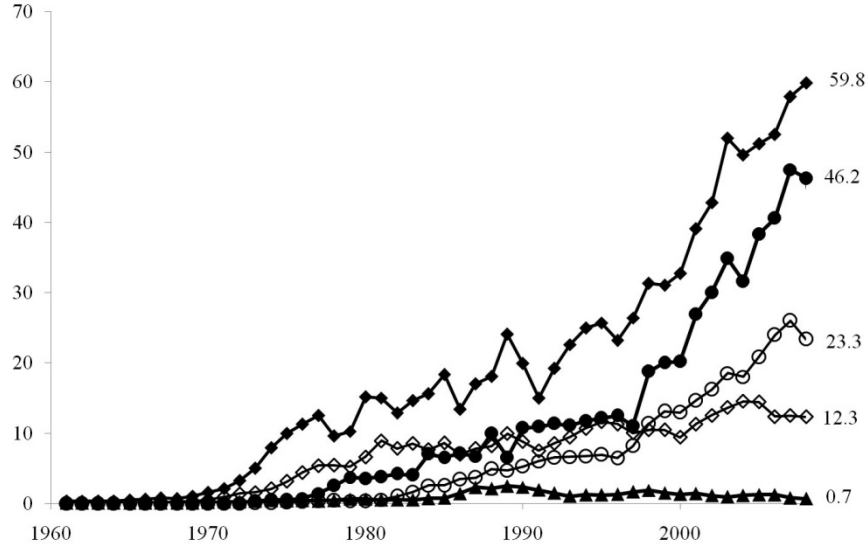

Figure 1. Soybean cake production in Brazil (♦), Argentine (●) and Europe (▲) from 1961 to 2009 and soybean cake export of Brazil (◊) and Argentine (○) from 1961 to 2008 in million tons (Food and Agricultural Organization of the United Nations., 2011).

Almost 80% of European animal feed stuff imports come from Argentina and Brazil (Eurostat, 2008), mostly in the form of soybean cake (30.6 million tons in 2008; Food and Agricultural Organization of the United Nations., 2011). A downside of this is the destruction of rainforests for soybean production (Ratter *et al.*, 1997). This exploitation of natural resources and dependency on imported protein feed ingredients can be reduced by local production of grain and forage legumes such as beans, peas, lucerne or clovers.

Lucerne and clovers are common feed ingredients for ruminants and lucerne in particular has been enhanced by intensive breeding programs which have produced high yielding varieties. Legumes have many benefits such as improvement of soil fertility but also weaknesses. As most vegetable proteins, they suffer from low protein utilization by ruminants, with concomitant high nitrogen losses. Various techniques are available to decrease these losses caused by excessive protein degradation in the rumen. Heat treatment is an established method to reduce ruminal protein degradation and thereby improve protein utilization of soybean or rapeseed meal. Other promising techniques involve the use of high pressure, tannins or coating with xylose products (Randby, 2000; Henderson, 1993).

2.2 Tannins

Tannins are commonly associated with an astringent taste in *e.g.* red wines and some unripe fruits but are actually present in many plants (Robichaud & Noble, 1990). Bate-Smith (1973) described their purpose in plants as follows:

"From the biological point of view the importance of tannins in plants lies in their effectiveness as repellents to predators, whether animal or microbial. In either case the relevant property is astringency rendering the tissues unpalatable by precipitating proteins or by immobilizing enzymes, impeding the invasion of the host by the parasite".

Certain tannins have been described as nutritionally beneficial to humans and animals. Biological properties, *e.g.* antimicrobial, anti-viral, anti-tumor, anti-cancer, nematicidal or antioxidant have been attributed to certain tannins (Aron & Kennedy, 2008; Mueller-Harvey, 2006; Fukuda *et al.*, 2003; Min *et al.*, 2003; Reed, 1995).

2.2.1 Tannin chemistry

Tannins are oligomeric, polyphenolic compounds, often with high molecular weight, and accumulate in many plants as natural products of secondary plant metabolism (Caygill & Mueller-Harvey, 1999). They show great structural diversity among different plant species but one feature that most tannins have in common is that they precipitate protein. Tannins can be divided chemically into two important groups: the hydrolysable tannins and the condensed tannins (CT).

Hydrolysable tannins are polyesters of sugars (mostly glucose) and gallic or ellagic acids (Figure 2) and are generally considered detrimental to animal nutrition (Serrano *et al.*, 2009). Condensed tannins are polymers of flavan-3-ols (Figure 3). They form colorful anthocyanidins upon oxidative cleavage (heating in presence of acid) and are therefore also called proanthocyanidins. Each CT polymer can consist of a variety of flavan-3-ol subunits of which the most common are catechin and epicatechin or gallocatechin and epigallocatechin which form procyanidins or prodelphinidins.

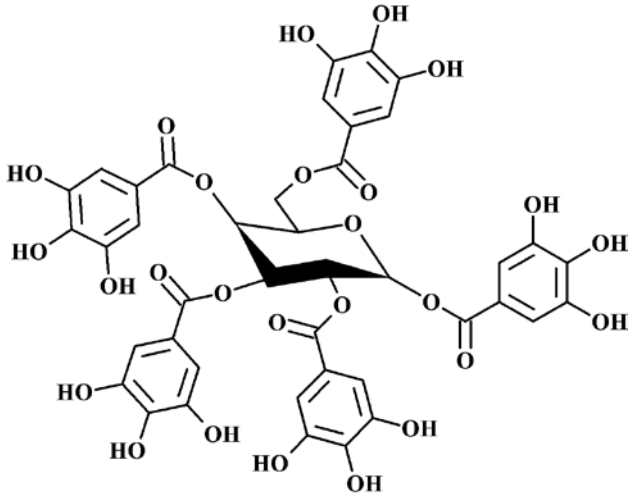

Figure 2. Structure of the hydrolysable tannin pentagalloylglucose.

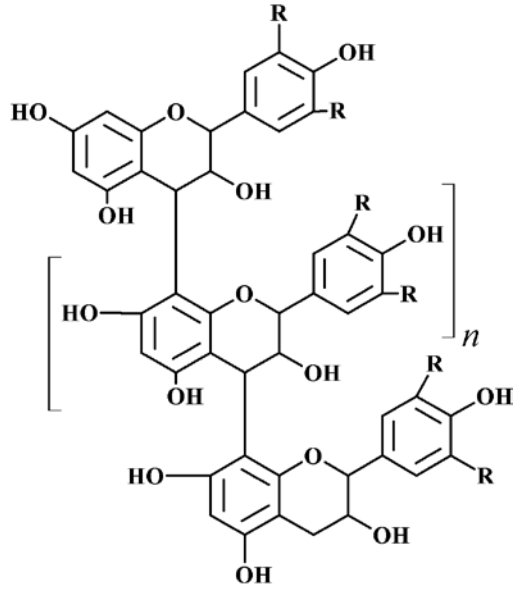

Figure 3. Possible structure of a condensed tannin with *n*+2 flavan 3-ol subunits.

The combination of different subunits, binding possibilities between the subunits and the degree of polymerization results in a large variety of polymers with different chemical and physiological properties (Figure 4).

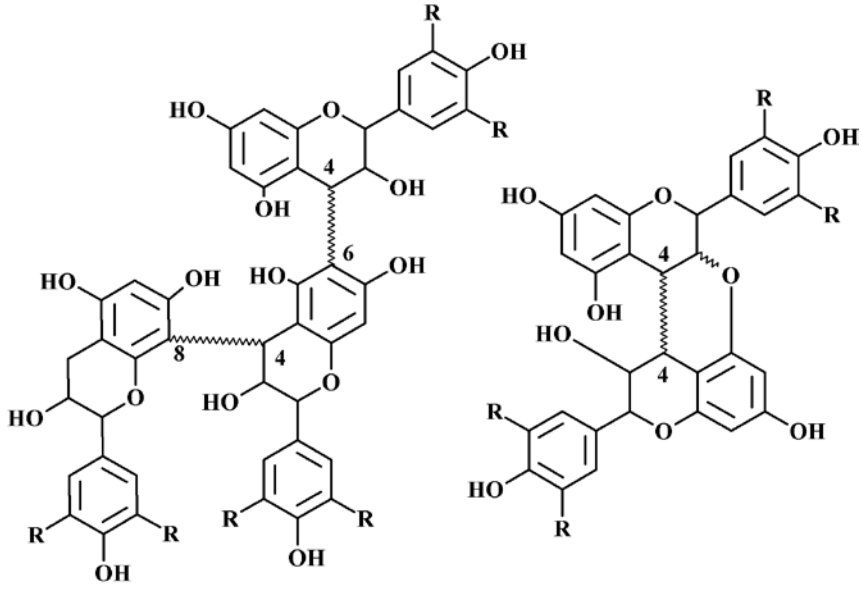

Figure 4. Possible condensed tannin bindings: B-type (C-C) binding on the left and an A-type binding (additional ether linkage) on the right.

2.2.2 Tannin-protein binding

The process of tannin-protein binding is complex and may depend on quantity, structure and size of both tannin and protein. Tannins have a high degree of hydroxylation that offers many possible sites for binding to proteins. Complexes are formed mainly via hydrogen bonding. Proline-rich proteins have been described as particularly reactive with tannins (Hagerman & Butler, 1981). Precipitation increases with size of the complex. This occurs as a result of more tannins binding to the protein, cross linking with other proteins, causing decreased hydrophilicity (Figure 5).

The polymer polyethylene glycol (PEG) has been shown to have high binding affinity to tannins and may even replace already tannin bound proteins (Jones & Mangan, 1977). This effect has been used to study tannin-protein binding (Silanikove *et al.*, 1996; Makkar *et al.*, 1993) or to alleviate detrimental nutritional effects caused by tannins (Priolo *et al.*, 2000).

The stability of tannin-protein bindings has been shown to be pH dependant (Oh & Hoff, 1987; Hagerman & Butler, 1981). They seem to be more stable close to the isoelectric point of the protein, *i.e.* for the most abundant plant protein Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) in a pH range from 3.5 to 7.0. The complexes are susceptible to release, particularly at low pH (Jones & Mangan, 1977).

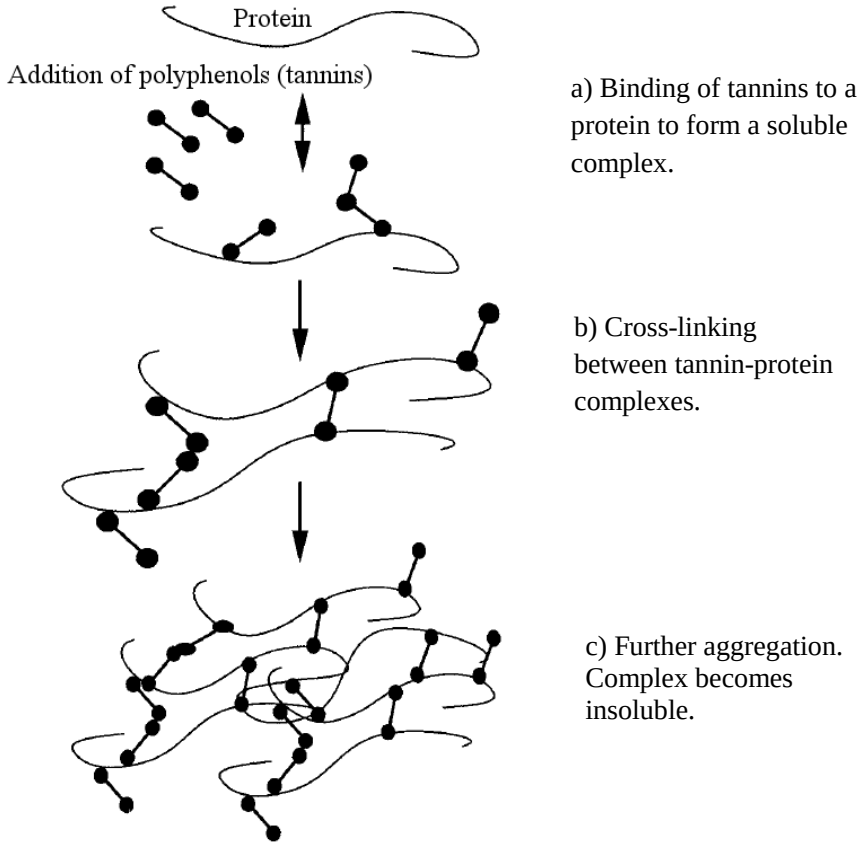

Figure 5. Depiction of a stepwise binding of hydrolysable tannins to a proline-rich protein (modified from Charlton *et al.*, 2002).

2.2.3 Tannins in ruminant nutrition

Tannins have for a long time been considered harmful in animal nutrition, particularly to monogastrics (Jezierny *et al.*, 2010). However, certain tannins can induce important benefits in ruminant nutrition and to the environment. When tannins bind to dietary proteins, they can become 'rumen-escape' proteins, which cannot be degraded by bacterial enzymes. These proteins pass on to the abomasum, where pH is around 2.5 and complexes may dissociate. Unless the stability of the complexes is too high, this can often lead to an improved protein utilization (Min *et al.*, 2003; Lowry *et al.*, 1996; Karnezos *et al.*, 1994; Oh & Hoff, 1987; Jones & Mangan, 1977).

Tannin-rich feeds can reduce wastage of N by reducing de-amination of plant protein in the rumen and subsequent losses in the urine. This results in a shift from urea to fecal N losses, which is considered beneficial from an environmentally point of view as fecal N is considerably less mobile in the soil (Patra, 2010; Theodoridou *et al.*, 2010; Min *et al.*, 2003). Urinary N in the

form of urea can be converted to N oxides and mainly to NH_3 (Vallejo *et al.*, 2006; Bussink & Oenema, 1998). Moreover, tannins also seem to reduce methanogenesis in ruminants (Patra & Saxena, 2010). This is potentially important because methane production by domestic ruminants is believed to be a major contributor to anthropogenic greenhouse gas emissions.

2.3 Sainfoin

2.3.1 General information on sainfoin

Sainfoin (Figure 6) is a perennial, frost and drought resistant forage legume that belongs to the subfamily Faboideae. In French, sainfoin means “healthy hay” and the denomination *Onobrychis viciifolia* means “devoured by donkeys”. Both names describe the positive characteristics accredited to this plant by farmers already hundreds of years ago. The German word Esparsette refers to its resemblance with *Vicia* or *Hedysarium* (Weigand, 1854).

Sainfoin was introduced from South Central Asia in the fifteenth century and reports of widespread cultivation date back to the sixteenth century (Hayot Carbonero *et al.*, 2011; Ambrosoli, 1997). However, during the early 1970´s, at a time of increasing use of artificial fertilizers, sainfoin quickly disappeared from European landscapes (Rochon *et al.*, 2004). Seeds of traditionally grown local sainfoin cultivars are therefore difficult to obtain or unavailable today.

Environmental issues and energy costs, associated with fertilizer production and application, are nowadays moving the focus to sustainable resources for animal feeding. The promising properties of sainfoin such as N-enrichment of the soil and improvement of protein utilization in ruminants are economically important and meet the demand of low energy input farming systems.

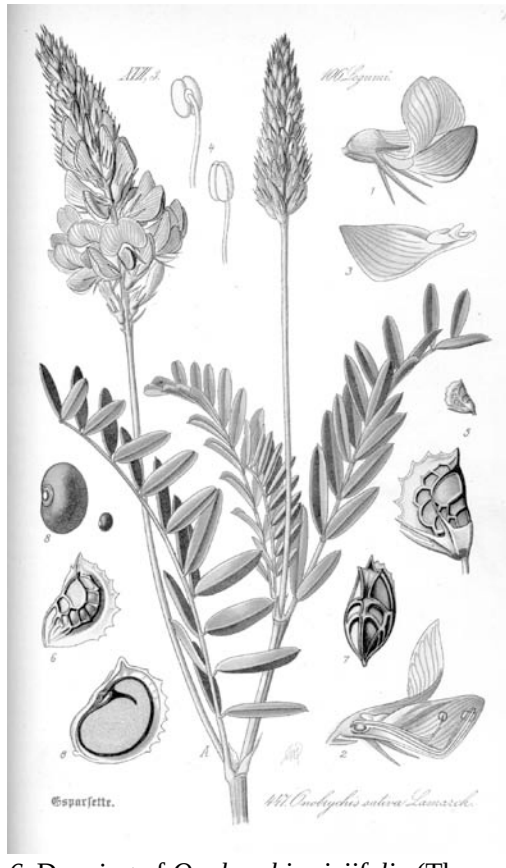

Figure 6. Drawing of *Onobrychis viciifolia* (Thome, 1885).

2.3.2 Cultivation and agricultural performance of sainfoin

Sainfoin grows best in the temperate climate zones on dry soils at a pH above 6.5 (Neuhoff & Bücking, 2006). A long tap root makes the plant drought resistant. However, establishment of the plant can be difficult due to its poor competitiveness. Long experience and studies in the UK show that sainfoin is susceptible to weeds and, therefore, herbicides are recommended at first trifoliate stage, if sown as a pure stand. In mixtures with grasses or on organic farms, spraying of herbicides is not an option. Meadow fescue or timothy are recommended companion species. Ryegrass can also be used, but less competitive, late tetraploid varieties should be used in combination with sainfoin. Once established, some varieties of sainfoin can persist for many years. Sainfoin can also be undersown to spring cereals at 50 kg of husked seeds per hectare (Cotswold Seeds Ltd., 2011).

The first cut usually gives high yields and is suitable for making hay or silage while the re-growth is often poor and better for grazing. Some varieties

of sainfoin can reach a height of up to one meter or more and with increasing maturity, the plant develops thick stems (personal observation). Sainfoin should therefore be harvested at an early flowering stage. According to GRIN (National Germplasm Resources Laboratory, 2011) there are 197 known accessions, which can be subdivided into common and giant types. The common types normally persist longer while the giant types show higher yields but are less persistent. Yields of up to 9 t dry matter (DM)/ha for the first cut and a total annual harvest of 15 t DM/ha have been recorded under experimental conditions (Koivisto & Lane, 2001). However, sainfoin generally gives lower yields than lucerne but can be grown as an alternative to lucerne in drier areas (Hayot Carbonero *et al.*, 2011).

2.3.3 Sainfoin in ruminant nutrition

Domestically grown protein sources are increasing in importance for animal feeds, particularly in organic farming. Sainfoin has been described as a beneficial fodder for horses and ruminants and, despite its high tannin content, it is highly palatable (Khalilvandi-Behroozyar *et al.*, 2010; Häring *et al.*, 2008; Fraser, 2000). Nutritional benefits of sainfoin have been reported in the form of decreased ruminal protein degradation (Broderick & Albrecht, 1997) and better wool growth, higher milk yield and decreased parasite establishment in the rumen (Heckendorn *et al.*, 2006; Paolini *et al.*, 2004).

Similar to other forage legumes that contain CT, sainfoin does not cause bloat in ruminants. Bloat can occur during feeding of legumes which are high in easily degradable proteins such as young lucerne or different varieties of clover. A buildup of froth in the rumen which decreases gas release is the consequence and can cause animal death. (McMahon *et al.*, 1999; Majak *et al.*, 1995; Cole *et al.*, 1945).

2.4 Conservation of forage

Hay and silage making are common practices to conserve forage. Conservation is necessary in areas where production of forage is not possible during the whole year due to cold winters or drought periods. Forage for traditional hay production is cut and sun dried on the field to reduce molding and subsequent nutrient losses. Drying also results in some loss of nutrients due to plant enzyme activity, growth of microorganisms and particle loss, mainly in the form of leaves. These losses can be minimized by rapid and professional forage handling. However, successful hay making is mainly dependant on periods of stable, dry and warm weather conditions which are largely beyond the control of the famer. Ensiling reduces farmer dependency on extended periods of dry

weather, as compared to hay making. Ensiling has therefore become increasingly popular (Van Os, 1997).

Ensiling is the process of storing crops in silos or bales under anaerobic conditions and controlled fermentation in such a way that minimum losses of nutrients occur. Wilting of the crops to reduce water content is often recommended to reduce nutrient loss by effluents and also in order to facilitate a desirable fermentation process. As with the conservation of hay, silage production is accompanied by nutrient losses. Severe and common problems are fungal growth. Fungi cause silage deterioration, resulting in DM loss and sometimes formation of mycotoxins. The majority of molds are obligate aerobes. Therefore, an oxygen-free environment is an efficient way to inhibit their growth. This can be achieved by thorough compaction and sealing of the silage in order to minimize contact to air. Once remaining oxygen in a silo or a bale is consumed, and provided that sufficient levels of sugars are present, conditions are favorable for anaerobic fermentation by lactic acid bacteria, clostridia and enterobacteria (Buxton *et al.*, 2003).

Clostridia and enterobacteria have major impact on deterioration and loss of nutrients. Fermentation end-products of lactic acid bacteria, in the form of lactic and acetic acid, lower pH to a point where bacteria other than lactic acid bacteria cannot survive (McDonald *et al.*, 1991). Silage additives such as acids and their salts are used to rapidly lower initial silage pH. Also inoculation with lactic acid bacteria can be useful to speed up initial fermentation rate. A pH below 4 is desirable for wet crops. However, final pH will eventually depend on the availability of sugars, buffering capacity of the crop and its DM content.

Proteins are susceptible to enzymatic degradation by plants and microorganisms during the conservation process. Proteolytic plant enzymes are activated directly after cutting and are difficult to control. The soluble protein fraction tends to be quickly transformed into peptides and AA´s. In the silo, clostridia and enterobacteria decarboxylate and/or deaminate AA´s. This results in a decreased nutritive value and a loss of energy by the formation of hydrogen, CO_2 and NH_3. Silage effluents increase with water content of the crop and can pollute ground water (McDonald *et al.*, 1991; Muck, 1988). A concomitant increase of butyric acid and biogenic amines also has an impact on feed intake and animal performance (Huhtanen *et al.*, 2003; Van Os, 1997; Huhtanen *et al.*, 1993; Muck, 1988; Bender & Bosshardt, 1939).

Most ensiling problems can be met by good ensiling practices. They include reduction of field losses by rapid transport from field into the silage containment, reduction of respiration by airtight silage sealing and by wilting to a higher DM level (Buxton *et al.*, 2003; McDonald *et al.*, 2002).

2.5 *In vitro* protein degradation assays

Knowledge of protein degradation in ruminant feeds is required to compose balanced diets. Controversy exists about the validity of protein degradation assays. *In vivo* feeding studies *e.g.* with markers and digestive tract collections may be more accurate but are costly, labor intensive and time consuming. Evaluations of *in vivo* measurements and other methods, primarily the *in sacco* method by Ørskov and McDonald (1979), have been performed (Norman *et al.*, 2010; Hedqvist & Udén, 2006; Sehgal & Makkar, 1994; Arieli *et al.*, 1993). The *in sacco* method is based on the incubation of feed containing small porous bags in the rumen of a fistulated animal. However, this method has been questioned because of several theoretical and practical drawbacks and its variability (Dewhurst *et al.*, 1995; Madsen & Hvelplund, 1994; Spencer *et al.*, 1988).

In search for substitutes for the *in sacco* method, several *in vitro* methods have been investigated. These involve use of ^{15}N, buffer solubility of feed components or incubation in bacterial enzyme solutions or rumen fluid (RF). These methods have both advantages and disadvantages. The gas *in vitro* protein degradation assay originated by Raab *et al.* (1983) and the inhibited *in vitro* (IIV) method by Broderick (1987) were used in this thesis.

2.5.1 Gas *in vitro* protein degradation by Raab

This method involves the measurement of gaseous end-products along with the formation of NH_3 during fermentation in RF. Ammonia is the product of AA deamination in the rumen and its level is related to the resistance of feed proteins to ruminal degradation and to the use of NH_3 by bacteria for synthesis of bacterial protein. Ammonia from feed protein degradation must, therefore, be corrected for bacterial protein synthesis. If incubations with multiple levels of carbohydrates are done, NH_3 evolution at zero bacterial growth can be estimated from the Y-intercept of the regression of NH_3 level on gas production (Figure 7). The intercept represents the theoretical amount of NH_3, which would have been produced from feed protein degradation at zero bacterial growth. This value divided by the amount of protein N incubated yields an estimate of *in vitro* crude protein degradability (IVDP).

A drawback of the method is that RF also contains protozoa which digest bacterial protein and interfere with the assumption that NH_3 derives only from dietary protein degradation.

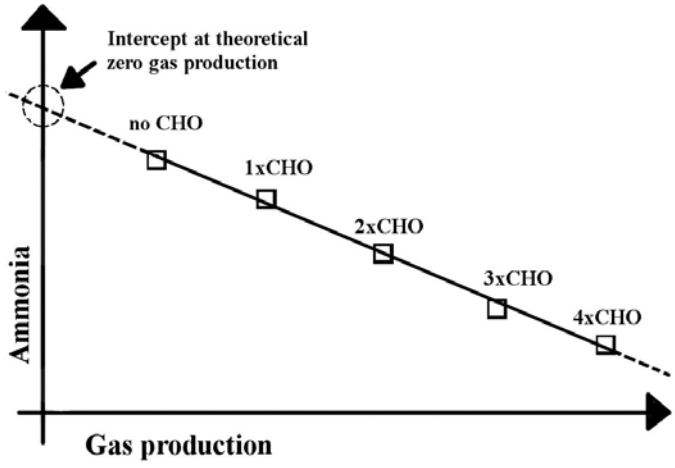

Figure 7. Calculation of *in vitro* degradability of crude protein of a feed incubated with four levels of carbohydrates (CHO).

2.5.2 The inhibited *in vitro* method by Broderick

The method is based on the measurement of AA´s and NH_3 as an indicator of feed protein degradability. Plant material is incubated in RF from fistulated cows for four hours. Amino acids and NH_3 content are measured every hour. Inhibitors of protein synthesis (hydrazine sulfate and chloramphenicol) are used to inhibit microbial proliferation. Therefore, contents of AA´s and NH_3 in the media are a direct measure of protein degradation. This method is useful for short but not long term incubation since microbial growth is inhibited and the population will eventually die.

3 Aims of the thesis

The overall aims of this thesis were

- the evaluation of tannin effects on protein degradation during ensiling and ruminal fermentation,
- to improve an *in vitro* protein degradation assay and
- the evaluation of a novel technique to measure protein precipitation by tannins.

The specific objectives can be divided into:

1. Silage fermentation:
   a) effects of different sainfoin DM levels,
   b) effects of PEG and a commercial silage additive and
   c) effects of high acidification of sainfoin during ensiling.
2. Rumen fermentation:
   a) effects of tannin content of different sainfoin varieties and
   b) improving the gas *in vitro* protein degradation assay by defaunation of the inoculum to inhibit N recycling by protozoa.
3. Protein precipitation:
   effects of sainfoin tannin molecular structure and protein type on protein precipitation by residual protein size screening.

4 Material and methods

4.1 Sainfoin varieties and their origin

Sainfoin varieties in Paper I were Cotswold Common, Teruel and Reznos. They were harvested in Zaragoza, Spain in April/May 2007. Wilted and un-wilted material was frozen and sent to the Kungsängen Research Centre, Uppsala, Sweden. Sainfoin varieties for Papers II & IV (Table 1) were harvested in August 2008 at the National Institute for Agricultural Botany (NIAB) in Cambridge, UK.

4.2 Ensiling procedure

Frozen sainfoin was cut into small pieces and dried to 50% DM (Paper I) or 40, 50 and 60% DM (Paper II). Plant material was treated with PEG or Promyr (Paper I) or with formic acid (Paper II) and ensiled in 100 ml mini-silos, closed airtight and stored in a dark room at 20°C. Promyr consisted of a solution of >750 g/kg formic acid and sodium-formates in solution and <250 g/kg propionic acid.

The silos were opened after 60 days and half of the content was frozen for further AA and NH_3 analysis. The other half was freeze dried for *in vitro* experiments and DM, total N, buffer soluble N (BSN), non-protein N, and tannin content were determined.

Table 1. *Available sainfoin and birdsfoot trefoil varieties for Paper I and II produced by NIAB.*

	Sainfoin *Onobrychis viciifolia*		
1	Ambra	25	Fizes
2	AR-111	26	Giant
3	Bivolari	27	Hampshire Common
4	Buciansky	28	Korunga
5	Cotswold Common-1	29	Line 107
6	Cotswold Common-2	30	Miatiletka
7	CPI 63750	31	Nova
8	CPI 63753	32	Palio
9	CPI 63758	33	Perly-1
10	CPI 63767	34	Perly-2
11	CPI 63810	35	Premier
12	CPI 63820	36	Rees "A"
13	CPI 63838	37	Simpro
14	CPI 63840	38	Somborne
15	CPI 63841	39	Sparceto
16	CPI 63854	40	Taja
17	CPI63752	41	Teruel
18	CPI63761	42	Tu86-43-03
19	CPI63808	43	Tu86-43-03
20	CPI63815	44	Unspecified-1
21	CPI63825	45	Unspecified-2
22	CPI63826	46	Visnovsky
23	Dnepropetrovsk	47	Wkt 10
24	Dukorastushchii	48	247
	Onobrychis antasiatica		
49	Akhurian-107	50	Sisiani Local
	Birdsfoot trefoil		
1	Grassland Goldie	2	Grassland Maku

4.3 Chemical procedures

4.3.1 N-fraction analysis

Total N content was determined by the Kjeldahl procedure (Nordic Committee on Food Analysis, 1976). Buffer soluble N was measured after one hour incubation in a borate-phosphate buffer (pH=6.75) according to Licitra (1996).

Non-protein N was determined after precipitation of the borate-phosphate buffer extract with trichloroacetic acid by the Kjeldahl procedure. Ammonia-N and AA-N were analyzed using phenol-hypochlorite and ninhydrin, respectively, on a Technicon Auto Analyser (Broderick & Kang, 1980). Leucine was used as a standard for AA´s and ammonium sulphate for NH_3-N.

4.3.2 Tannin measurement methods

HCl/butanol method (Papers I and II)

Triplicates of 50 mg freeze dried sample were weighed into 30-ml polypropylene test tubes. Five ml of HCl/butanol reagent (HCl:butanol=1:20) were added to the samples and they were incubated in a water bath set at 100°C for 60 min. Tubes were cooled to 20°C and absorbance read on a spectrophotometer at 550 nm. For Paper I and II, tannin values were expressed relative to the absorption of a purified sainfoin extract (var. Cotswold common-1) in g/kg DM. Extractable and protein bound tannins were measured according to Terrill *et al.* (1992). Tannin values for Paper IV were reported on the basis of absorbance units at 550 nm per 50 mg DM ($AU_{(550)}$).

Radial diffusion assay (RDA; Paper IV)

Protein precipitation capacity using bovine serum albumin (BSA) was analyzed by the RDA according to Hagerman and Robins (1987). Protein precipitating capacity using Rubisco was analyzed according to Giner-Chavez *et al.* (1997). Rubisco was extracted from fresh spinach leaves according to a simplified protocol from Andersson *et al.* (1983). The protein extract was purified on a Sephadex G-25 column (60 x 8 cm). Rubisco was detected on a spectrophotometer at 280 nm and confirmed by gel electrophoresis.

4.3.3 Measurement of tannin structure-dependant protein binding (Paper V)

In vitro incubations of protein- and tannin extracts

Condensed tannin extracts were dissolved in H_20, resulting in four different solutions with concentrations 0.2 to 2.0 g/l. Proteins were BSA, rapeseed protein and Rubisco. A volume of 100 µl of each protein solution was mixed with 100 µl of each CT solution in 1.5 ml plastic tubes. The tubes were incubated for 15 min at 39°C. After incubation, the tubes were centrifuged at 10 000 x *g* for five min at room temperature. A CT free mixture was incubated as a reference sample. The remaining soluble proteins in the supernatant were size screened and quantified by micro gel electrophoresis.

Lab on a chip electrophoresis

The method is a micro gel-electrophoresis in chip format by Agilent Technologies (Agilent Technologies, Palo Alto, USA). Proteins samples were mixed with a buffer from the Agilent kit and dye labeled. Samples were pipetted into openings on the chip which are connected to channels filled with a chromatography gel. The sample is sieved through the gel and size separated by an Agilent 2100 Bioanalyzer (Agilent Technologies, 2006). The standard sample buffer of the kit contained an internal standard determining the relative concentration of the sample, between a lower an upper marker, based on the upper marker concentration. Proteins were detected by laser-induced fluorescence. Each chip can be used for analysis of ten individual samples. Data was analyzed by the Agilent Expert 2100 software (Agilent Technologies, 2009).

4.4 *In vitro* protein degradation assay

4.4.1 Inhibited *in vitro* method (Paper II & IV)

The IIV method by Broderick is used to compare treatment and variety effects to sainfoin protein degradation. The method estimates feed protein degradation from the formation of NH_3 and AA´s. Plant material is incubated in buffered RF which was pre-incubated with carbohydrates to increase microbial activity. Hydrazine sulphate and chloramphenicol are added to the RF prior to incubation in order to inhibit microbial growth. Rumen fluid is continuously sampled during a four hour incubation and AA-N and NH_3-N levels are measured.

The fraction of undegraded protein N at the respective time point i ($FUD_{(i)}$) is calculated as:

$FUD_{(i)} = 1 - FD_{(i)}$

where $FD_{(i)} = (NH_3\text{-}N_{(i)} + AA\text{-}N_{(i)})/N_{(sample)}$, $N_{(sample)}$ = N content of the incubated feed sample; $NH_3\text{-}N_{(i)}$ and $AA\text{-}N_{(i)}$ are blank corrected with respective blanks.

4.4.2 Gas *in vitro* protein degradation (Paper III)

A modification of the gas *in vitro* method by Raab *et al.* (1983) was tested to measure protein degradation (see chapter 2.5.1). The modifications were as follows: Rumen fluid was pre-incubated with carbohydrates to equilibrate NH_3 levels and enhance microbial activity. Protozoa were removed from RF by centrifugation for five minutes at 3000 x *g* prior to pre-incubation. Gas production was measured during incubation simultaneously in the incubations vessels by a fully automated system (Cone *et al.*, 1996). Ammonia was

measured at different intervals according the modification by Karlsson *et al.* (2009) during 23 hours. After incubation, IVDP was calculated.

5 Results

5.1 Paper I

Results of Paper I describe the effect of ensiling with and without a commercial silage additive or PEG on N-fractionation in sainfoin. Variety Cotswold Common had the highest total N content (22.6 g/kg DM). Total N and BSN were increased by wilting. Silage BSN was influenced by variety and treatment with PEG and wilting. Polyethylene glycol treatment doubled BSN from 296 to 596 g/kg N in direct cut silage and from 323 to 676 g/kg N in wilted silages. Highest non-protein N contents were observed for PEG treated Cotswold Common. Grass clover silage BSN levels were high and unaffected by PEG treatment. Extractable tannins were twice as high in ensiled sainfoin as in fresh plants. Protein bound tannins increased with ensiling relative to total tannin content. Only a weak correlation between extractable tannins in fresh forage and silage BSN was observed (R^2=0.45).

Varieties were not different in total N but Reznos had higher BSN and the lowest ratio of non-protein N to BSN. Silage N and BSN concentrations in wilted, Promyr treated sainfoin were 1.06 and 1.16 relative to direct cut silage. Treatment with Promyr decreased BSN particularly in direct cut silages.

5.2 Paper II

Results from Paper II describe the N-fractionation in sainfoin at various DM levels and formic acid acidification. Silage pH was 3.67 and 4.76 for low and high DM contents, respectively. The lowest silage pH was measured at the highest acidification level and lowest DM content, whereas the highest pH was measured in the non-acidified, high DM silages (Figure 8).

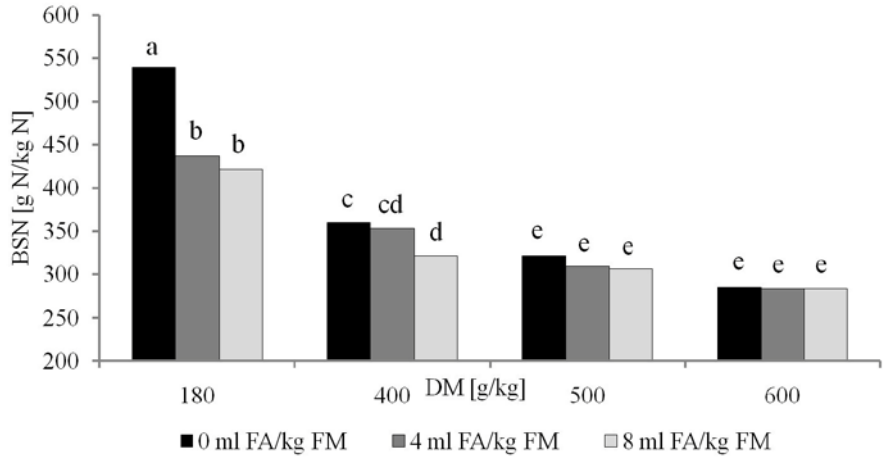

Figure 8. Buffer soluble N (BSN) in acidified (ml formic acid (FA)/kg fresh matter (FM)) and non-acidified silages at different dry matter (DM) levels. Values with different letters differ at a 5% level.

The proportion of BSN to total N was influenced by both formic acid addition ($P<0.01$) and DM level ($P<0001$). Moderate and high formic acid addition decreased BSN ($P<0.01$) in silages whereas no difference was observed between moderate and high formic acid addition ($P<0.22$). The lowest levels of BSN were observed in samples dried to 600 g/kg DM ($P<0.002$). Increasing formic acid addition from medium to high had no effect on non-protein N, AA-N and NH_3-N ($P<0.21$). Rates of *in vitro* degradation did not change between treatments as measured by the IIV method.

5.3 Paper III

Results from Paper III describe effects of defaunation of RF on GP and NH_3 evolution in the Raab protein degradation assay. Defaunation of RF by centrifugation was successful without compromising bacterial activity. Ammonia-N concentrations in defaunated RF after 23 h in blanks and soybean meal were 32 and 173 mg/l compared to 221 and 377 mg/l in untreated rumen fluid ($P<0.002$). Gas production in defaunated RF in blank and soybean meal samples were 105 and 166 ml compared to 127 and 190 ml in untreated RF ($P<0.008$). Ammonia-N concentration in untreated RF blanks increased after 10.5 h of incubation ($P<0.004$) from a minimum at 4.5 h, while defaunated RF blanks did not increase until after 17.5 h ($P<0.024$) after first reaching a minimum at 6 h.

Estimates of IVDP of soybean meal samples were lower in defaunated RF (0.55) than in untreated RF (1.03) and exceeded the theoretical maximum of 1.0 for IVDP. *In vitro* digestibility of crude protein was negative at hours 6 and 7.5 in untreated RF when blank corrected.

5.4 Paper IV

Results of Paper IV describe the total N, BSN, DM, tannin contents and IIV protein degradation of 38 different sainfoin and two birdsfoot trefoil varieties. Total N was normally distributed and ranged from 15.8 to 31.7 g/kg DM. Buffer soluble N ranged from 102 to 335 g/kg N. Tannin measurements of different sainfoin varieties by the RDA showed that protein precipitation of BSA was closely correlated to precipitation of Rubisco (Figure 10, Panel A; R^2=0.929). Correlation of the HCl/butanol method and the RDA were 0.587 and 0.521 for BSA and Rubisco. When birdsfoot trefoil was excluded from the calculation, R^2 increased to 0.713 (BSA) and 0.759 (Rubisco).

Correlations between IIV protein degradation values and different tannin measurements methods were weak. Values for the fraction of undegraded protein at incubation hour four ($FUD_{(4)}$) ranged from 0.515 to 0.773. Buffer soluble N and $FUD_{(4)}$ had an R^2 of 0.433 (Figure 10, Panel B). Average protein degradation of sainfoin was 36%. There was no correlation between $FUD_{(4)}$ and tannin contents using HCl/butanol method or RDA (Figure 10, Panels C and D).

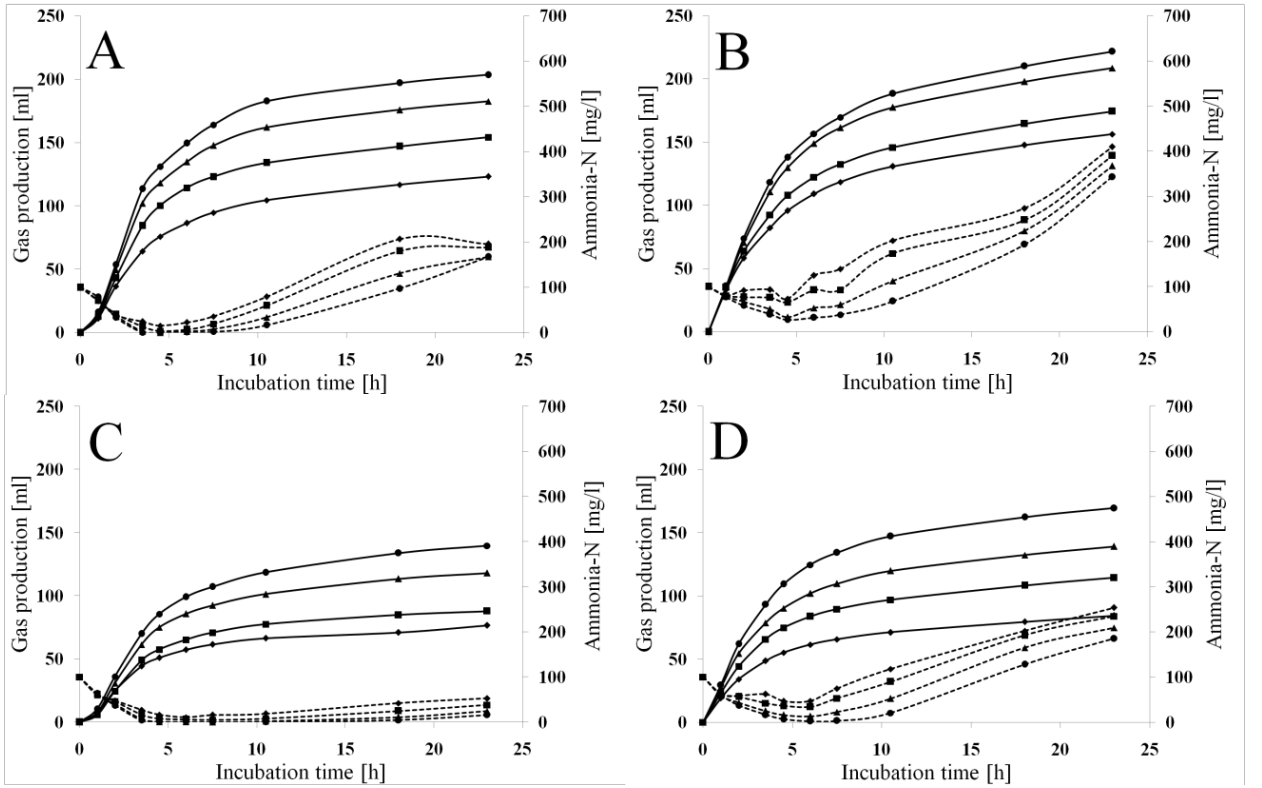

Figure 9. Gas production (solid lines) and ammonia-N concentration (dashed lines) of soybean meal (Panels A and B) and blanks (Panel C and D) using defaunated (Panels A and C) or untreated rumen fluid (Panels B and D) during an incubation period of 23 h. Carbohydrate additions were 100 (♦), 200 (■), 300 (▲) and 400 (●) mg/flask.

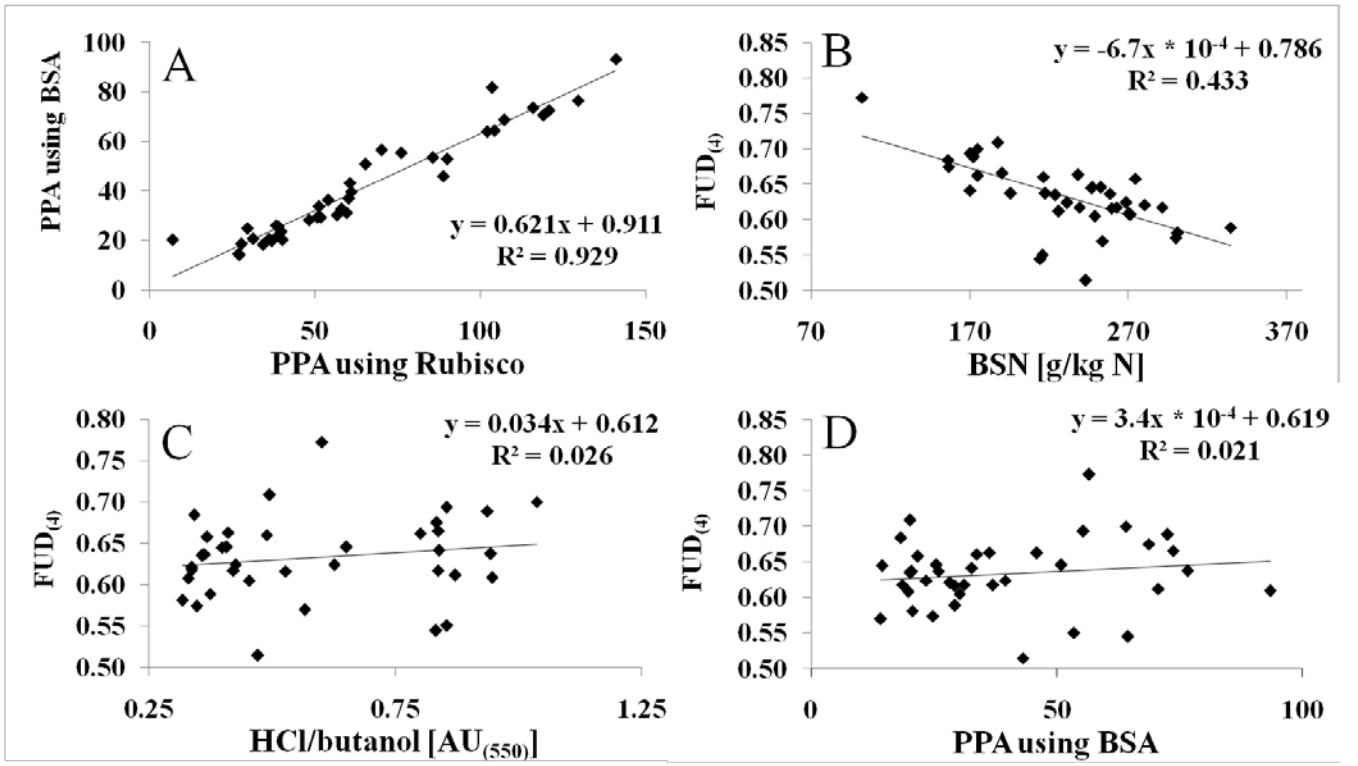

Figure 10. Regressions between protein precipitation area (PPA; mm^2) of sainfoin extracts using Rubisco *vs.* BSA (Panel A), undegraded protein fraction at fourth incubation hour ($FUD_{(4)}$) *vs.* buffer soluble N (BSN; panel B); $FUD_{(4)}$ *vs.* HCl/butanol response (Panel C) and $FUD_{(4)}$ *vs.* PPA using BSA (Panel D).

5.5 Paper V

Results of Paper V show the analysis of residual BSA and Rubisco protein after incubation with four different CT types. Proteins were quantified and size screened. Molecular CT type structures are described in Table 2. Differences in precipitation among proteins (P<0.001) and CT type (P=0.003) were observed with only a tendency for a protein*CT interaction (P<0.073). Pairwise comparisons (not shown) indicated differences mainly between CT type A and B (P<0.002), B and D (P<0.065) and for BSA compared to Rubisco and rapeseed protein (P<0.001). Increasing CT concentration increased protein precipitation (P<0.001). Protein (P=0.325) and CT type (P=0.993) did not influence binding specificity relative to protein size. Protein fraction size binding was influenced only by CT concentration (P<0.001).

Table 2. *Structural characteristics of condensed tannin types A, B, C and D.*

Tannin type	mDP		Flavan-3-ol subunit fractions			Fractions of intra-molecular flavan-3-ol binding			
	Mean	SD	PD	:	PC	SD	Cis	:	Trans	SD
A	32.3	1.4	71.7	:	28.3	0.3	75.6	:	24.4	1.9
B	76.5	0.4	86.5	:	13.5	1.6	72.5	:	27.5	7.3
C	16.0	0.8	86.3	:	13.7	0.2	77.3	:	22.7	2.6
D	18.9	0.7	68.3	:	31.7	0.4	84.1	:	15.9	1.0

mDP, mean degree of polymerization; PD, prodelphinidin; PC, procyanidin; SD, standard deviation.

6 Discussion

This thesis aims to answer questions about sainfoin tannin related nutritional characteristics affected by different DM stages, different acidification levels using different silage additives and ruminal protein degradation. A novel approach to elucidate tannin structure-dependant protein precipitation has been tested. Further, a methodological improvement for an *in vitro* ruminal protein degradation assay by defaunation of RF is presented.

6.1 Evaluation of treatment effects on sainfoin silage (Papers I and II)

Forage proteins for ruminants are subject to degradation during harvesting, wilting, conservation and ruminal fermentation. Ensiling is the dominating conservation method today, but good ensiling practices are necessary to minimize degradation losses. These practices may include wilting, treatment with silage additives to lower pH or induce lactic acid fermentation by inoculation. Although a low pH is critical to make good silages from wet crops, it might also dissociate tannin-protein complexes. Dissociation of these complexes already during ensiling would compromise formation of rumen escape protein that can improve protein utilization in high producing dairy cows.

Legumes are generally low in water soluble sugars and have high buffering capacity which make ensiling difficult (Buxton *et al.*, 2003). Nevertheless, all sainfoin silages showed good ensiling characteristics as no molds or yeasts were visible and NH_3 levels were low. Nitrogen values of varieties grown in Spain (Paper I) and in the UK (Paper II) were between 20 and 26 g N/kg DM and within the range of earlier observations (Scharenberg *et al.*, 2007b; Turgut & Yanar, 2004; Fraser, 2000). After forage treatment with an acidifying silage additive prior to ensiling in Paper I, pH was reduced to approximately 4. After

ensiling for 60 day, pH decreased to 3.90 (Paper I) and to 3.67 (Paper II). Earlier tannin-protein binding studies suggest that complexes can be released at pH of 2 to 3 (Jones & Mangan, 1977) or below 3.5 (Perez-Maldonado *et al.*, 1995). Silage pH did not drop beyond 3.67 in Paper II and release of tannin-protein complexes did not seem to occur, judging from the low BSN contents. In Paper I and II, lowering pH with silage additives resulted in decreased BSN and non-protein N values. This indicates that in sainfoin the addition of high levels of formic acid does not lower pH beneath a level where tannin-bound proteins are released.

Amounts of extractable and protein bound tannins in Paper I were similar to earlier reports on wilted and ensiled sainfoin (Scharenberg *et al.*, 2007a; Scharenberg *et al.*, 2007b; Hristov & Sandev, 1998). The proportion of extractable tannins to total tannins was 0.6 in fresh material and decreased to 0.3 in silages. It can be hypothesized that the remaining extractable tannins did not bind due to their chemical structure or due to a physical restriction. Extractable tannins in ensiled material were lower in the PEG treated silage ($P<0.05$) indicating a higher affinity of tannins to PEG than to protein. A similar effect of PEG on BSN was also observed by Jones and Mangan (1977). Polyethylene glycol has a high affinity to tannins and may either inhibit the binding of tannins to proteins or exchange proteins that are bound to tannins with PEG. Makkar *et al.* (1995) showed inhibition of enzymes and microorganisms by tannins which was released with PEG treatment.

Only a weak correlation between tannins levels and N solubility was observed. This was particularly the case for the tannin levels in un-ensiled sainfoin and BSN in the corresponding silage. Similarly, Vitti *et al.* (2005) concluded in an experiment with various tanniniferous legumes that neither high nor low tannin concentration could be attributed to positive or negative nutritional characteristics. A non-linear relationship between BSN and sainfoin and birdsfoot trefoil tannins analyzed by the RDA was observed by Hedqvist (2004). Nevertheless, the results indicate that interpreting breakdown of protein in silages require more information than merely concentrations of extractable and protein bound tannins measured by the Terrill method.

An indirect measurement of protein protecting capacity of tannins could be related to the PEG treatment. Similarly low values for BSN and non-protein N in Cotswold Common and Teruel without PEG, probably relates to the high binding strength of their tannins to proteins. After treatment with PEG, values for BSN and non-protein N remained lower in Teruel than in Cotswold common and may indicate a higher affinity of Cotswold Common tannins to PEG. However, this interpretation of indirect tannin effects by PEG is not practical for evaluating forage for ensiling because ensiling is time consuming.

It is necessary to develop a more suitable method that can link tannin characteristics to nutritional effects. In Paper V, tannin structure characteristics are discussed further.

6.2 Evaluation of tannin measurement methods relative to protein degradability (Paper IV)

A multitude of tannin measurement methods exist and many of them are summarized in the tannin handbook by Hagerman (2002) but there is no single established standard. A combination of tannin measurement methods as tested by Wisdom *et al.* (1987) or McAllister *et al.* (2005) appear promising but time consuming since biological activity, tannin molecular weight and chromophore production have to be measured.

Giner-Chavez *et al.* (1997) suggested a methodology where plant leaf protein is used instead of BSA to test for protein precipitation capacity since non-plant proteins might not be meaningful for ruminant nutrition studies. In Paper IV, Rubisco and BSA were used in protein precipitation studies on 38 different varieties of sainfoin. Precipitation capacity in the RDA using BSA and Rubisco were highly correlated (R^2 = 0.929) which suggests that BSA and Rubisco have similar binding properties to sainfoin tannins. Results from this study also suggest that protein size is not a determining factor for binding to tannins of sainfoin, at least not for the globular proteins Rubisco and BSA. Attempts to use the fibrous protein gelatin for an RDA to study the effect of tertiary protein structure on binding behavior were not successful.

An R^2 of 0.95 between the HCl/butanol method and the RDA (using BSA) for measuring tannin in six birdsfoot trefoil varieties was observed by Hedqvist *et al*. (2000). In Paper IV, an R^2 of 0.521 was observed for sainfoin and birdsfoot trefoil combined but when birdsfoot trefoil was excluded, R^2 increased to 0.759. This was probably due to more tannin variation between different plant species than within one species. The lower R^2 of sainfoin tannins compared to birdsfoot trefoil tannins could probably be due to a high variation in the content and structure of sainfoin tannins as previously reported by Scharenberg *et al.* (2007a) and Gea *et al.* (2011). High molecular weights of up to 28 100 Da and a high PD to PC ratios were observed by Marais *et al.* (2000) and Jones *et al*. (1976). Furthermore, Foo *et al.* (1982) hypothesized that sainfoin tannins vary significantly among cultivars and seasons and may also have an unusual tertiary structure. However, this is of little relevance in the face of a very low correlation between these tannins methods and protein degradation *in vitro*. The fact that tannin content and protein precipitation only had an R^2 of 0.587 is disconcerting. It is likely that the molecular structure of

tannins is the main reason for the poor relationship between precipitation capacity and tannin content as suggested by Horigome *et al.* (1988) and Min *et al.* (2003). It might also be that factors other than CT might have an effect. With a smaller sample than in the present study (4 *vs.* 38 samples), Hedqvist *et al.* (2000) also found a high correlation (R^2=0.93) when *in vitro* protein degradation of different birdsfoot trefoil varieties was compared with tannin concentration measured by the HCl/butanol method and the RDA.

6.3 Evaluation of tannin structure dependant protein binding by Lab on a chip gel electrophoresis (Paper V)

An influence of structural characteristics on protein binding was observed in Paper V. Mean degree of polymerization, intra-molecular cis and trans binding and ratio of procyanidin to prodelphinidin were variables correlated to protein binding and precipitation. Differences were also observed due to increasing concentration and type of CT and protein. Increasing CT concentration increased precipitation of higher molecular weight proteins relative to smaller. However, it was not possible to relate structural characteristics of CT types to effects on protein precipitation. Selective inhibition and stimulation of certain rumen bacteria by tannin of different structure or sources were observed by Sivakumaran (2004) and McAllister (2005), respectively. A tendency to an interaction between protein and tannin type was also seen in Paper V.

It is concluded that none of the examined characteristics could predict protein degradation. Other structural factors of CT, apart from those investigated, such as flavanoid linkage patterns or extent of branching, could be more decisive for effects on protein precipitation (Clausen *et al.*, 1990).

6.4 Evaluation of the Raab *in vitro* protein degradation assay (Paper III)

Current prediction methods for feed protein quality by *in vitro, in sacco* and *in vivo* methods are unsatisfactory. They show a critical number of unpredictable variables. Paper III aimed to improve the Raab *in vitro* protein degradation assay. *In vitro* methods have the advantage that they are less costly, time consuming, ethically less controversial and more repeatable than *in sacco* methods. The modified Raab method tested in this study seemed promising but shows a number of practical shortcomings that need to be addressed. For instance, the quality of RF as inoculum is of high importance as shown by Broderick *et al.* (2004) and was recently addressed by improvements to increase bacterial activity and uniformity of RF by Karlsson *et al.* (2009).

Rumen fluid is not only a habitat for bacteria but also for other organisms such as protozoa. The metabolism of these organisms changes the nutrient pool of the media. Protozoa engulf and digest bacteria by phagocytosis, particularly in times of carbohydrate shortage (Bonhomme, 1990). Digestion of bacteria by protozoa releases NH_3 into the surrounding media which conflicts with the assumptions that are made for IVDP calculations (see Chapter 2.5.1).

Experiments to reduce recycling of bacterial protein by protozoa by defaunation were attempted in Paper III. It was shown that RF can be defaunated without compromising bacterial activity and that recycling of NH_3 can be almost entirely halted. Blank incubations with and without defaunated RF demonstrated that blank corrections of untreated RF incubations cannot be correct. Ammonia levels in blank samples were partly even higher than in soybean meal samples since an earlier depletion of carbohydrates in blank samples leads to an increase in protein degradation. Also reproducibility was increased by incubation with defaunated RF.

A problem which was discovered with the use of defaunated RF was NH_3 depletion at early incubation hours. This resulted in a distortion of IVDP values. Addition of known amounts of NH_3 at the beginning of the incubation could have the potential to improve IVDP calculation for short incubation times. Nevertheless, the Raab *in vitro* method is based on a promising concept and has the potential to become a reliable method for feed protein evaluation.

7 Conclusions

Sainfoin is a forage legume that shows promising ensiling characteristics at different DM stages and using commercial silage additives.

Protein degradation during ensiling and *in vitro* RF incubations was low due to the protein sparing effect of tannins.

Sainfoin tannins were shown to be still active when the silage is ensiled with high amounts of acid as protein degradation was not increased at a pH as low as 3.67.

Varying molecular tannin structure within the sainfoin species affects protein precipitation of proteins *in vitro*. However, mean degree of polymerization, ratio of cis to trans binding and ratio of PC to PD impacts protein size related binding but are not good predictors of protein precipitation.

The implications of protozoal metabolism on the Raab *in vitro* RF gas production method were described. Modifications performed to improve the method were successful. Ammonia background levels were reduced by defaunation of RF while gas production as a measure of fermentation activity was maintained.

8 Future research

Legumes are likely going to play a more important role in European agriculture in the future. The project “Re-invention of sainfoin” is one of many different pillars on which the strategy to improve food production with less energy input could be based.

Momentarily, the supply of seeds of formerly local sainfoin varieties is restricted in many areas. Future work has to test suitability of available varieties to specific sites where seeds of local varieties are no longer available anymore. Experiments testing the agricultural performance over longer periods are necessary.

There is a need to understand the molecular structure-dependant binding of tannins to proteins. Other structural characteristics than those tested in this study, could be more decisive. Analytical and biochemical test have to fully clarify mode of action of tannin-protein binding first, before further nutritional research on tannin specific effects on proteins are performed.

Feed protein degradation assays for ruminants are unsatisfactory. The *in vitro* gas production technique by Raab is based on an promising concept. Future research has to address shortcomings such as unreliable IVDP values in the beginning of the incubation due to depleted NH_3 levels. Further improvements should consider addition of sufficient NH_3 to the inoculum to prevent substrate shortage.

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