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Les effets des tannins condensés du sainfoin (*Onobrychis viciifolia*) sur sa digestion et sa valeur nutritive

The effects of condensed tannins in sainfoin (*Onobrychis viciifolia*) on its digestion and nutritive value

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HEALTHYHAY
Marie Curie Research Training Network

In memory of my father who taught me that if you have a dream you should follow it…

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Table of contents

TABLE OF CONTENTS

Acknowledgement

1. **INTRODUCTION** 11
   - 1.1 Background 13
   - 1.2 The project "HealthyHay'' 15
     - 1.2.1 Objectives of the project 15
     - 1.2.2 Organization of the project 15

Aim and experimental strategy of the thesis 17

2. LITERATURE REVIEW 21

I. Tannins 23

1. Definition 23
2. Hydrolysable tannins 23
3. Condensed tannins 25
- **3.1 Nomenclature and primary structure of condensed tannins** 25
- **3.2 Structural properties of condensed tannins** 26
  - **3.2.1** Content 27
  - **3.2.2** Biological activity and mean degree of polymerisation (mDP) 27
  - **3.2.3** Prodelphinidin (PD):Procyanidin (PC) ratio 29
  - **3.2.4** Stereochemistry 29
- **3.3 Occurrence, location and role of tannins in plant tissues** 29
- **3.4 Biosynthesis of condensed tannins** 30
- **3.5 Factors affect the biosynthesis of condensed tannins** 31
  - **3.5.1** Related to plant 31
  - **3.5.2** Related to environment 33
- **3.6 Toxicity of condensed tannins** 34
- **3.7 Preparation of plant material and extraction of condensed tannins** 34
- **3.8 Inactivation of condensed tannins** 35
- **3.9 Interaction of tannins with other molecules** 37
  - **3.9.1** Proteins 38
  - **3.9.2** Carbohydrates 41
  - **3.9.3** Metal ions 41
  - **3.9.4** Enzymes and bacteria 41
- **3.10 Analytical methods of condensed tannins** 46
  - 3.10.1 Colorimetric assays 42
  - 3.10.2 Protein precipitation assays 43

3.10.3 Mixed assays 44
3.10.4 Structural characterization assays 44
4. Nutritive effects of condensed tannins 47
4.1 Voluntary intake and palatability 47
4.2 Nitrogen metabolism 48
4.2.1 Effect of condensed tannins on ruminal protein degradation 48
4.2.2 Effect of condensed tannins on N absorption and amino acid supply to small intestine 49
4.2.3 Effects of condensed tannins on urea cycle 51
4.2.4 Effect of condensed tannins on nitrogen excretion and retention 51
4.3 Effect of condensed tannins on ruminal fermentation 53
4.4 Carbohydrate metabolism 54
4.5 Animal performance 55
4.5.1 Wool growth and live weight gain 56
4.5.2 Milk and meat production and quality 57
4.5.3 Animal productivity 59
4.6 Animal health 59
4.6.1 Bloat 59
4.6.2 Gastro-intestinal parasites 59
4.7 Environmental impact of condensed tannins 61
4.7.1 Nitrogen emission 61
4.7.2 Methane emission 61

II. Sainfoin 63

1. Agronomical value 63
1.1 Identification 63
1.2 Morphology 63
1.3 Climate and soil requirements 64
1.4 Use of sainfoin 65
1.4.1 Pasture 65
1.4.2 Conserved forage: hay and silage 65
1.5 Dry matter (DM) yield 66
1.6 Diseases and insects 66

2. Nutritive value 67

2.1 Chemical composition 67
2.2 Content and structure of sainfoin CT 67
2.3 Palatability and voluntary feed intake 68
2.4 Rumen degradation 68
2.5 Digestion in small intestine 69
2.6 *In vivo* digestibility 69

2.7 Nitroge retention and excretion 68
2.8 Animal performance 69
2.9 Animal health 69
2.9.1 Bloat 69
2.9.2 Anthelmintic properties 70
Concluding remarks 72

3. EXPERIMENTAL WORK 73
3.1 Part 1 75
***In vivo* and *in situ* effect of condensed tannins in fresh sainfoin on digestive and nitrogen balance and on ruminal and intestinal digestion parameters.**
3.2 Part 2 113
Effects of condensed tannins of sainfoin on the production and composition of fermentation gases.
3.3 Part 3 151
How the preservation method modified the content of condensed tannins and their effects on the sainfoin digestion and its nutritive value.
3.3 Part 4 187
Evaluation of sainfoin varieties in term of their chemical composition, content, structure and biological activity of condensed tannins. Comparison with lucerne.
4. DISCUSSION 219
I. Content, structure and biological activity of condensed tannins in sainfoin 222
1. Methods used for the quantification of CT 222
2. The structure of CT and its relationship with biological activity 224
3. Relationship between CT characteristics and morphological or chemical composition of sainfoin 227
4. Change in content, biological activity and structure of CT with phenological stage 229
II. Effects of condensed tannins on digestive utilisation of sainfoin 232
1. Organic matter digestibility and dry matter disappearance (DisDM) in the rumen 233
2. Nitrogen digestion 234
3. N excretion and methane emissions 239
III. The nutritive value of sainfoin 242
1. Energy value 242
2. Nitrogen value 247
CONCLUSIONS AND PERSPECTIVES 257
PUBLICATIONS 263
REFERENCES 263

Chapter 1

Introduction

1.1 Background

Ruminants are the only animals that are able to make good use of the considerable quantities of proteins present in forage plants, while at the same time utilising the energy supplied not only by the cell sugars but also, and above all, by the cellulose and hemicelluloses of plant cell walls. Forage plants are a major source of dietary proteins for ruminants, providing about 85% of their needs. Even though the utility of fodder legumes has been recognised for several centuries, crop areas under pure fodder legumes have continued to shrink in France and elsewhere in Europe since the period 1950-1960. This regression is the result of a combination of several factors that have marked recent decades:

- The specialisation of agricultural regions, causing the regression of livestock farming in crop-growing areas.
- The low price of soy cake protein on the European market at world rates and the low price of fossil energy, ensuring the availability of low-cost chemical fertiliser.
- The huge development of maize silage, combining full mechanisation right through from sowing to distribution, easy conservation and a high energy value stable in time, allowing considerable simplification of forage systems wherever maize can be grown with high, regular yields.

In recent years the context has changed: agriculture must now simultaneously produce more to meet world food supply challenges, provide new products (e.g. agrofuels, green chemicals, environmental services), and also produce differently, with greater respect for the environment. The recent world food crises, caused in particular by the strong competition of agrofuels, have led to an increase in the prices of inputs, putting pressure on livestock farming and encouraging greater use of forage plants for ruminant feed.

At the international, European and French levels, several reports, directives, regulations and political initiatives (e.g. the French national summit on the environment) have pointed out the environmentally unfriendly aspects of ruminant farming. The sharpest criticism concerns greenhouse gas emissions, in particular methane in the herbivore sector, which contributes to global warming. There is also a need to limit the risks of pollution of water, air and soil, and to preserve non-renewable resources and biodiversity.

Ensuring a secure supply of food of animal origin through greater feed self-sufficiency of herds to offset risks incurred by having to purchase foods or fears aroused by genetically modified foods has prompted a greater development of forage for feeding ruminants. The development of quality-certified animal products is reflected in more precise requirements as regards modes of production and the origin of raw materials. This is the case for both organic farming and a growing number of controlled-origin or certified products.

Today the objective of livestock farmers is above all to improve forage self-sufficiency, nitrogen value of forages and resistance to drought, with a concern to limit recourse to inputs and

irrigation, while allowing for the impending scarcity of fossil fuels and meeting the expectancies of the public at large for a sustainable agriculture of quality that preserves natural resources.

Fodder legumes or dietary associations are ideal ways to approach all these goals. Although fodder legumes have many strengths, in particular their high nitrogen contents and their self-sufficiency for nitrogenous nutrition, the solubility of their foliar proteins is very high: this can lead to a marked degradation of these proteins in the rumen, culminating in ammonia loss in urine, which is also source of pollution. Only the proteins bound to cell wall constituents enjoy a degree of protection.

Another weakness of forage legumes lies in harvesting and conservation. Drying for hay takes longer than for grasses and so is more dependent on fine weather. Heavy losses of leaves often ensue. Ensilage of these nitrogen-rich and relatively sugar-poor forages can result in mediocre conservation and in particular degradation of proteins unless preparatory haymaking is thorough or preservatives are added. These proteins conserved by ensilage or haylage remain readily fermentable in the rumen of ruminants; they are converted into ammonia before being re-utilised by bacteria for the synthesis of new proteins if enough energy is available.

Although there are differences in solubility of proteins among species of legumes, searching the variability within species displaying the highest solubilities, such as lucerne, has not revealed any variation that could be exploited by genetics.

The presence of the enzyme polyphenol oxidase (PPO), contained in red clover, allows a marked reduction in non-protein nitrogen degradation in silage (Jones *et al*., 1995). The selection of forages with a high PPO activity might be a useful way to reduce protein losses in either silage or the rumen (Huygue *et al*., 2008).

Other solutions can be proposed to overcome this problem of excessive solubility (Huygue and Tabel, 2009). Among technological processes, dehydration modifies the spatial conformation of proteins by the effect of heat, thereby causing a marked reduction in their degradability, and making dehydrated lucerne a source of proteins of high dietary value. Haymaking and barn drying provide an intermediate degree of protein protection. Baling of half-hayed forage corrects its mediocre conservation while leaving the possibility of full haymaking when the weather permits it.

The use of forages containing condensed tannins (CT) is another solution. These accumulate in the vacuoles, and during mastication bind to feed or microbial proteins *via* hydrogen bonds, forming sparingly soluble complexes that are not degraded in the rumen. In the intestine, where the pH is lower, these bonds are cleaved and the proteins can be absorbed by the intestinal wall. For the same reasons tannin-rich legumes do not cause bloat. Many research studies on the value of tannin-containing forage have been carried out on birdsfoot trefoil (*Lotus corniculatus*), but this plant often has a low yield and the extent of its growth is linked to soil type.

Sainfoin is a legume containing condensed tannins that was commonly cultivated in Europe in earlier centuries. However, it has declined considerably since the massive advent of fertilisers in the 1960s and because of the difficulty growing it outside dry calcareous areas and its low perennity (2–3 years at most). It possesses many agronomic, nutritional and environmental qualities. In addition, its CT possesses, among other assets, anthelmintic properties of special interest to organic farming.

1.2 The project 'HealthyHay'

The HEALTHYHAY network brings together groups with a common interest and expertise in sainfoin, and is made up of teams with specific know-how who have well established track records in their research fields. HealthyHay is a unique approach, bringing together - for the first time-plant genetic resources in agriculture, nutritive science, veterinary science, environmental science, polyphenol and tannin chemistry, biochemistry and molecular biology. The combination of plant breeding with in-depth chemical, biological and molecular evaluations in order to improve veterinary, nutritive and environmental effects of an animal feed represents a completely novel interdisciplinary approach **(Figure 1).**

1.2.1 Objectives

This four-year project will tackle the scientific objectives in a multidisciplinary approach. The project contains 10 work packages covering research & development activities. This will contribute towards:

1 Developing a new source of home-grown protein,
2 Reducing environmental pollution from ruminant production systems (nitrogen and methane emissions),
3 Developing a fodder legume suitable for sustainable agriculture,
4 The foundation for new sainfoin breeding programmes,
5 Establishing a collection of the existing sainfoin germplasm and evaluating the agronomic, genetic, nutritive and veterinary properties of this germplasm collection,
6 Investigating the unique chemical, nutritive and veterinary properties of sainfoin and their modes of action,
7 Developing novel screening tools to underpin future sainfoin breeding programmes.

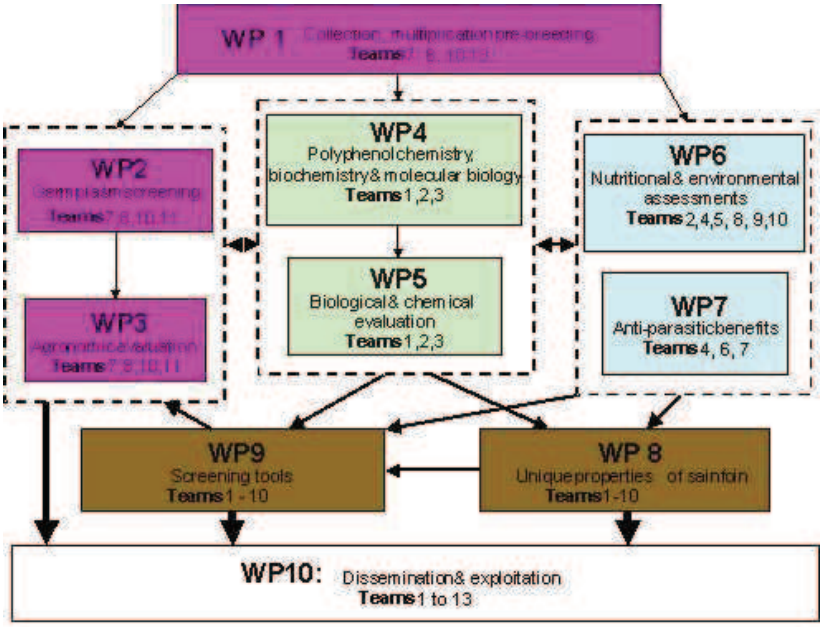


Figure 1: The work packages in the 'HealthyHay' project.

1.2.2 Organization

'HealthyHay' Marie Curie Research Training Network (RTN) brings together 13 teams from 10 countries in a multidisciplinary consortium:

Armenia	United Kingdom
Austria	Denmark
Greece	Spain
Sweden	The Netherlands
France	Germany

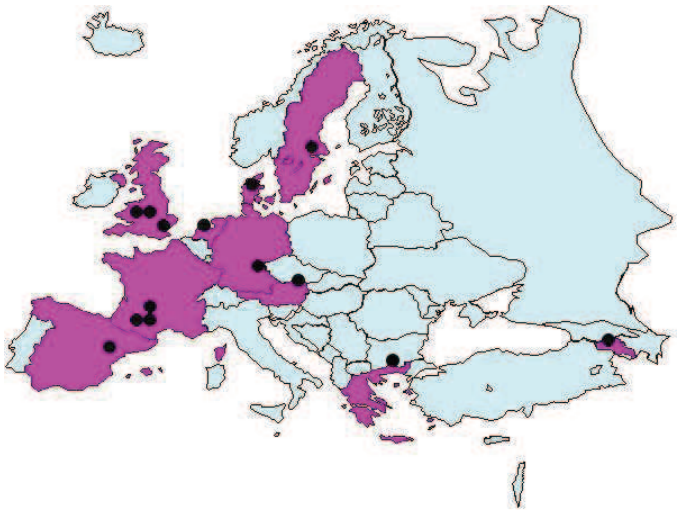


Figure 2: List of the countries participating in the project 'Healthy Hay'.

The research organisms participating in this network are: Technische Universität Wien (TUW), University of Reading (URE), Technische Universität München (TUM), INRA/ Ecole Nationale Vétérinaire de Toulouse (INRA-ENV), INRA/ Unité de Recherches sur les Herbivores (INRA-URH), Sveriges lantbruksuniversitet (SLU), Royal Veterinary and Agricultural University (KVL), National Agricultural Research foundation (NAGREF), Centro de Investigation y Tecnologia Agroalimentaria de Aragon (CITA), Wageningen Universiteit (WU), National Institute of Agricultural Botany (NIAB), Caussade Semences SA (CAUS), Cotswold Seeds Ltd (COTS), National Academy of Sciences of Armenia (NAS) **(Figure 2).**

Aim and experimental strategy of the thesis

This thesis is concerned with the investigation of the effect of condensed tannins (CT) on the nutritive value of sainfoin.

The aim of my thesis is to extend our knowledge of the nutritional value of the sainfoin forage legume and its environmental advandages when feeding to ruminants. In particular, the effects of condensed tannins (CT) were assessed for reducing nitrogen excretion and other gas emissions.
Agronomic and technological applications require that several factors be considered, e.g. the variation of the feed value due to the phenological stages of sainfoin, a range of preservation methods and varietal differences. We, therefore, have combined the study of CT effects with factors that vary in the feed valus in order to **address the following questions:**

1. **How are the structure, content and biological activity of the CT in sainfoin related to its phenological stage, vegetation cycle, preservation method and the variety studied?**

2. **According to each of these factors, what are the effects of CT in sainfoin on the digestive processes that determine its nutritive value and the environmental impact of its digestive utilisation?**

3. **What quantitative assessment can be made of the nutritive value of sainfoin**?

To address the first question, specific methods of analysis for CT and biological activity measurement have been implemented. In a joint work programme with the Chemistry and Biochemistry Laboratory, Reading University, and its head Dr. Mueller-Harvey, we opted for the thiolysis assay method, which provides both a quantitative measure of the CT, and qualitative

data on the main structural parameters that influence their activity. In addition, in this thesis we used the HCl-Butanol method, modified by Dr. Mueller-Harvey, to confirm the presence of CT in our sainfoin material. In our laboratory we used the radial diffusion assay (RDA; Makkar *et al*., 2000) which allows the biological activity of CT to be determined in terms of protein-binding capacity.

To address the second question, we elected to demonstrate the effect of sainfoin CT, which have been assessed by administrating polyethylene glycol (PEG) into the rumen in one group (PEG sheep) whilst a separate group of sheep received water (CT-acting group). PEG selectively binds with CTs preventing the CTS from binding plant proteins in the rumen so that effects of CT can be determined by comparing the CT-acting group with PEG sheep. To study the nutritive value and digestive processes we opted for an *in vivo* approach on the reference animal model for feed value studies, the male castrated sheep. This approach makes it possible to obtain overall balances at the whole animal scale (digestive balances and nitrogen losses) and to analyse the digestive processes by specific measurements in the different segments of the digestive tract (rumen and intestine). These last measurements used methods for the study of digestion *in situ* by the nylon bag technique. The *in situ* methods, like the *in vitro* method used to measure gas production, also enabled us to analyse the digestive processes separately for each of the two main parts of the plant, the leaves and the stems.

During our study, forages were distributed in a limited quantity per kg metabolic $BW^{0.75}$ to limit the variation in voluntary feed intake of sheep which could have affected the parameters of digestion.

Four experimental studies were conducted in this work, all of which help to answer the above questions. Each has been written up as a research paper.

In the first *in vivo* experiment we studied the effect of sainfoin CT at three phenological stages. This experiment tested the effect of fresh forage on the overall digestive and nitrogen balance of ruminants and on the ruminal and intestinal digestion parameters, using the *in situ* method (**PART 1**).

This *in vivo* study was combined with an *in vitro* study, which evaluated the same forages and tested the CT effects on the production and composition of fermentation gases (CO_2 H_2, O_2 and CH_4) **(PART 2**).

A second *in vivo* study was used the same sainfoin material as in Part 1, but preserved in the form of wrapped silage bales. This materialhad been harvested at the same phenological stages as the fresh forage. This study enabled us to assess how the preservation method modified CT

content and structures and their effects on the digestion of the forage and its nutritive value **(PART 3)**.

Lastly, we analysed the effects of CT content, structure and biological activity on the nutritive value (i.e. chemical composition and enzymatic digestibility) during the first and second vegetation cycles using three sainfoin varieties which had benn cultivated in the same location. The three varieties of sainfoin were compared with lucerne from the same location **(PART 4).**

For our experiments we used two sainfoin varieties (Ambra and Perly) that were already registered in the European catalogs and are officially recognised. We also used two other varieties (Esparcette and Villahoz) which are not registered. Esparcette (name used by Cotswold Grass Seeds) was a local variety from Ukraine. Villahoz is a cultivated type from a region in Spain (Burgos) where it used locally, being well-adapted to the climate.

In the last part, in a general discussion bringing together our results and critically comparing them with those of the literature, we highlight their contribution to a fuller understanding of the relation between the structure of CT and their nutritive value for ruminants, and we show how our findings offer answers to the three questions initially raised. We draw conclusions concerning the implications of our findings for a judicious choice of vegetation stage or preservation method of the forage to optimise harvesting conditions and ensure a high feed value for ruminants.

Chapter 2

Literature review

The aim of this chapter is to investigate the existing interaction of condensed tannins with other molecules and provide insight into some of the key factors that influence these interactions. In the light of this review an attempt has been made to highlight some of the detrimental and beneficial effects of condensed tannins reported in the literature in terms of ruminant nutrition. In the second part of this chapter characteristics and nutritive properties of sainfoin (*Onobrychis viciifolia*) will be described.

I. Tannins

1. Definition

The name tannin comes from the French *tan* meaning the bark of the oak and other trees used in tanning. The first person who used the term "tannin" was probably Seguin in 1796, describing the substance in oak galls responsible for the creation of leather (Zimmer and Cordesse 1996).

Tannins are phenolic plant secondary compounds, widely distributed throughout the plant kingdom, especially legumes and browse, with ranging molecular weights and complexity (Min *et al*., 2003b; Makkar, 2003).

One of the most satisfactory general definitions of tannins was proposed by Horvath (1981): "water soluble polymeric phenolics of sufficiently high molecular weight containing sufficient phenolics, hydroxyls and other suitable groups (carboxyls) to form effectively strong complexes with protein and other macromolecules under the particular environment conditions being studied".

The first classification of the tannins, based only on their chemical structure, was made in 1920 by Freudenber, who divided them into

- Hydrolysable tannins (HT) and
- Condensed tannins (CT).

2. Hydrolysable tannins

Hydrolysable tannins (HT) are relatively rare in nature or present in low amounts in plants (Ben Salem, 1998), but they are abundant in leaves, fruit pods and plant galls or dicotyledons, such as oak or chestnut (Kumar and D'Mello, 1995; Min *et al*., 2003b). The majority of the HTs are located in the cell fibre and the intracellular space (Terrill *et al*., 1992b; Frutos *et al*., 2002).

Some acacia species and a range of browse contain HT with up to 200 g/kg HT in their dry matter (Waghorn, 2008). Hydrolysable tannins are potentially toxic as pyrogallol their degradation product by ruminal microbes, is a hepatoxin and nephrotoxin. The toxic effect of HT is evident especially when large quantities are given to ruminants with insufficient time for microbial adaptation. It has also long been known that the toxicity of tannins is related to their

molecular size, since tannins with high molecular weights cannot be absorbed. This might explain the high toxicity of low molecular weight HT (500-3000) compared with the larger molecules of condensed tannins (1000-2000) (Reed, 1995; Norton, 1999; Frutos *et al*., 2004).

The molecules of HT comprise a central sugar moiety (in most cases glucose). The hydroxyl groups of these carbohydrates are partially or totally esterified with phenolic groups, such as gallic acid or hexahydroxydiphenic acid (**Figure 3**). Through the process of hydrolysis the HT release the phenolic acids. If the acid component is gallic acid the compounds are called gallotannins. If it is ellagic acid (formed after hydrolysis) they are called ellagitannins (Ben Salem, 1998; Serrano *et al*., 2009). We note that some species produce either gallotannins or ellagitannins, while others produce complex mixtures containing gallo-, ellagi- and condensed tannins (Mueller-Harvey, 2001).

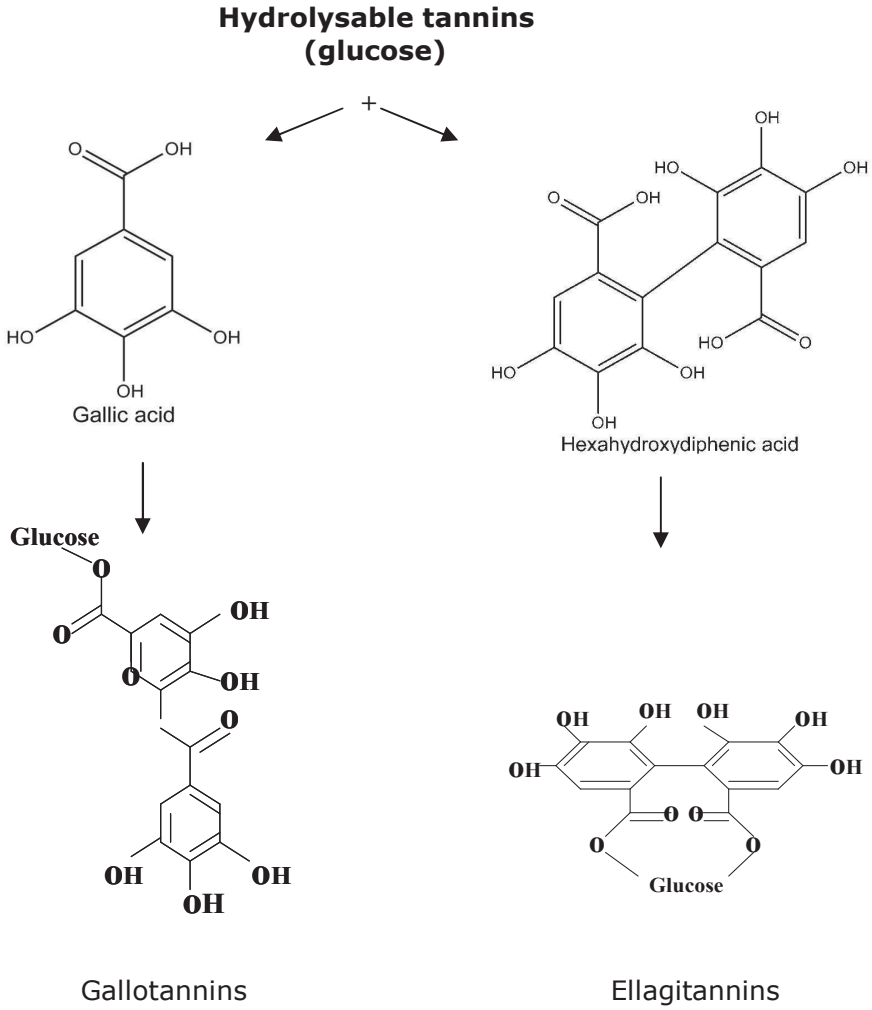


Figure 3: Structure of the hydrolysable tannins (HT).

3. Condensed tannins

3.1 Nomenclature and primary structure

Condensed tannins (CT), known as proanthocyanidins, are polymers of flavanoid units (flavan-3-ols) linked *via* carbon-carbon bonds that are not susceptible to anaerobic enzyme degradation (Waghorn, 2008), and produce anthocyanidins upon treatment with acidic alcohol (Porter *et al*., 1986). The monomeric units can be linked by C4/C8 or C4/C6 interflavanoid linkages, which affect the shape of the CT polymer chain (**Figure 4**). Different combinations of OH and H groups in these monomeric units, at positions R1, R2 and R3, lead to different classes of polymers, *e.g.*, prodelphinidins (PD) and procyanidin (PC) (**Table 1; Figure 4b**). Prodelphinidin is the generic term for a polymer containing gallocatechin *(trans*) or epigallocatechin (*cis*) subunits. Procyanidin refers to a polymer of catechin (*trans*) and epicatechin *(cis)* subunits (Schofield *et al*., 2001; Cheynier *et al*., 1999) (**Figure 5**).

Table 1: Different classes of polymer due to different combinations of OH and H groups

Unit	R1	R2	R3
(+)-catechin	OH	H	H
(-)-epicatechin	H	OH	H
(+)-gallocatechin	OH	H	OH
(-)-epigallocatechin	H	OH	OH

CT polymers in temperate forages may range in chain length from dimers to over 20 flavanol units, and there are usually several flavanol-3-ol structures within each polymer (Waghorn, 2008). Structural variations within CT include: the number of hydroxyl groups on the "A" and "B" rings of the flavanol units; positions of the hydroxyl groups; flavanol units at carbons 2,3 and 4 of the "C" ring; position and type of linkages between flavanol units and number of flavanol units (Mueller-Harvey and Mc Allan, 1992; Schofield *et al*., 2001; Waghorn, 2008). At position R2 there is an OH group, sometimes esterified to gallic acid. The presence of gallate esters in tannins can change their biological properties (Hagerman, 1992; Schofield *et al*., 2001).

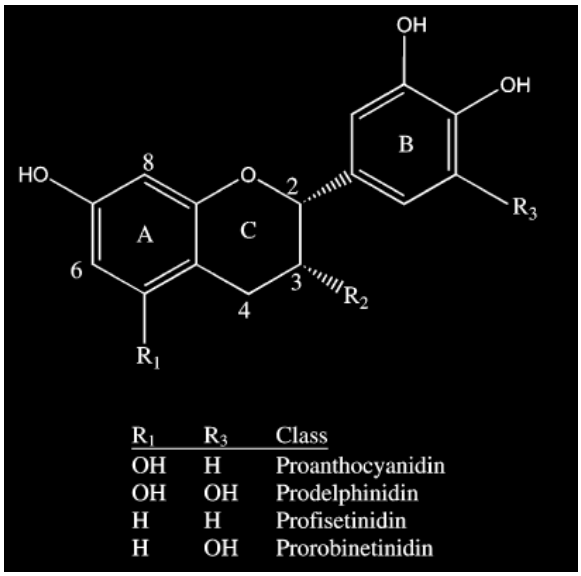


Figure 4: The basic repeating unit in condensed tannins. If R1= R2 = OH, R3 = H, then the structure is that of (-) epicatechin. The groups at R1 and R3 for other compounds are indicated below the structure. R2 =O gallolyl in the catechin gallates.

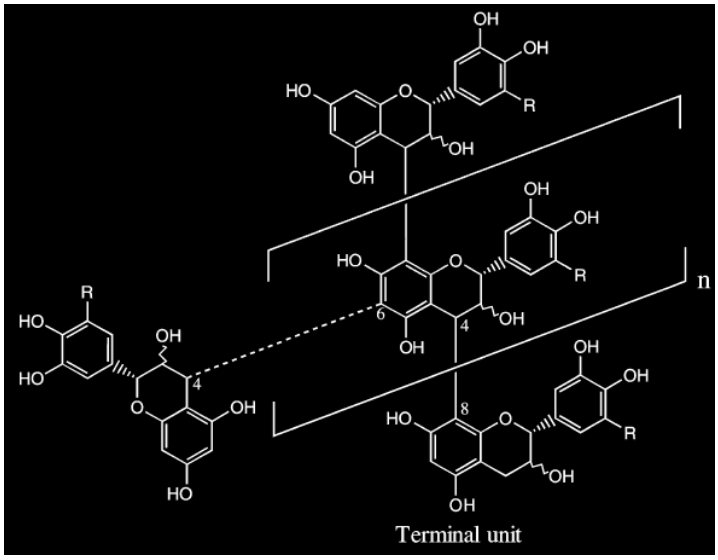


Figure 5: Model for condensed tannin. If R = H or OH then the structure represents a procyanidin or prodelphinidin. The 4-6 linkage is an alternative interflavan bond. The terminal unit is at the bottom of this multi-unit structure.

3.2 Structural properties of condensed tannins

The protein-binding effects of tannins are influenced by many factors, such as CT content, biological activity (astringency), mean degree of polymerisation (mDP) (*i.e.*, mean molecular size), prodelphinidin/procyanidin (PD:PC) ratio, *cis:trans* ratio, the ratio of proteins to tannins,

protein structure and amino acid composition, etc. (Spencier *et al*., 1988; Silber *et al*., 1998; Frazier *et al*., 2003; McAllister *et al*., 2005) .

Only few attempts have been made to evaluate how the structure of tannins influences the biological activity of tannin (Clausen *et al*., 1990).

3.2.1 Content

Dietary condensed tannins (CT) are defined in terms of content, as well as either beneficial or detrimental impacts on animal performance. However, the value of CT depends on the analytical technique (Hedqvist *et al*., 2000) and the animal and plant species (Waghorn and McNabb, 2003). Some leguminous species such as sainfoin (*Onobrychis viciifolia*), sulla (*Hedysarium coronarium*) and *Lotus* (*pedunculatus* and *corniculatus*) contain a quantity of CT of about 20 to 50 g/kg DM. Species of pine (*Pinus*), wattle *(Acacia)*, oak *(Quercus)* and apple or strawberry *(Rosaceae)* contain high CT quantities greater than about 50 gCT/kg DM (Ben Salem, 1998).

From observations on lotus species the highest content of tannins are in leaves (Gebrehiwot *et al*., 2002). Despite several studies on CT contents and structures of these species (Foo *et al*., 1982; Hedqvist *et al*., 2000; Min *et al*., 2003b; Meagher *et al*., 2004) there are only few reports characterising sainfoin CT.

Trials with sainfoin containing about 80 gCT/kg DM have demonstrated that it has high nutritive value for sheep, whereas diets including carob (*Ceratonia silique*) pulp and containing only 25 gCT/kg DM depressed growth rates (Priolo *et al*., 2000). Barry and McNabb (1999) advised that dietary contents of <50 gCT/kg DM would be beneficial for animal health and nutrition. However, these recommendations originated mainly from feeding trials with *Lotus* species and may not be applicable to other feeds (Waghorn and McNabb, 2003; Mueller-Harvey, 2006). The CT from *Lotus corniculatus* is associated with better animal performance than that from *Lotus pedunculatus* (Waghorn *et al*., 1999), although the content of CT in *Lotus corniculatus* is about half that in *Lotus pedunculatus* (Terrill *et al*., 1992b*).*

An 'optimal' type or content of CT will vary with the time over which it is fed and according to whether the CTs are intended to benefit nutrition or welfare.

3.2.2 Biological activity (astringency) and mean degree of polymerisation (mDP)

The number of monomeric units (*i.e*., degree of polymerisation) can vary and influence the biological activity of tannins (Clausen *et al*., 1990). Molecular size of CT varies widely according to factors such as forage species and plant tissue (Cano *et al*., 1994; Foo and Porter, 1980) and through the growth season (Butler, 1982). The average chain length of CT ranges from two flavan 3-ols units in barley seeds to 20 or 25 in *Lotus peduncularus* and sainfoin leaves (Mueller-Harvey and McAllan 1992). Generally, we distinguish oligomers, consisting of 2 to 10 monomer units, from polymers, with more than 10 monomer units.

Astringency is a measure of protein binding (and precipitating) capacity, or capacity to cause changes in enzyme activity. CT in *Lotus corniculatus* has been shown to be less astringent than that in *Lotus pedunculatus*. The CT in *Lotus corniculatus* comprises mainly catechin and epicatechin monomers (procyanidin), whereas *Lotus pedunculatus* shows a predominance of epigallocatechin units (prodelphinidins) (Waghorn, 2008).

The biological activity of CT or 'astringency' is related to the mDP (*i.e*., the molecular size of tannins) (Porter and Woodruffe, 1984; Sarni-Marchado *et al*., 1999; Cheynier, 2005; Frazier *et al*., 2010). Golstein and Swain (1963) suggested that the decline in astringency of fruits on ripening is due to an increase in the degree of polymerisation of tannins. Other studies on forages found that CT astringency increased with the mDP up to a point, but at the upper end of the molecular weight scale, tannins appeared to be less able to interact with proteins, meaning that larger tannins are less astringent (Mangan 1988; Aerts *et al.,* 1999). Osborne and McNeill (2001) evaluated the abilities of differently sized condensed tannins from the genus *Leucaena,* a fodder tree legume*.* They concluded that the higher the molecular weight of the CT, the greater was the tendency to precipitate protein. The tendency to interact with protein increases as the molecular weight of the tannin increases, as observed also by Verzele *et al*. 1986.

Kumar and Horigone (1986) studied the CT from black locust (*Robinia pseudo acacia*) leaves, concluded that the increase of the molecular weight of tannins leads to a decrease of their solubility and to an increase of their protein precipitation capacity. Lower molecular weight CTs can be more reactive, precipitating more protein than higher molecular weight CTs (Butler 1982). Some reports indicate that tannins greater than 3000 Da will precipitate, because CTs with molecular weights greater than 3000 Da become insoluble, and are often more closely linked to the structural tissues of plants, inactivating them (Salunkhe *et al*., 1990).

By contrast, some studies report that smaller tannins have higher biological activities because they are better able to interact with proteins. Jones *et al*., 1976 reported that the leaves of sainfoin contained tannins with fairly high molecular weights (17000-28000) were less efficient in precipitating blood proteins than the tannins of *Lotus* species with molecular weights of 6000-7100 Da.

For tanniniferous plants, tannin astringency also increases with their PD contents (Jones *et al*., 1976; Aerts *et al*., 1999). As the leaf mature, the active sites on tannins for binding with proteins become fewer in number or tannin molecules become too large to interact effectively with the protein (Makkar *et al*., 1988; De Freitas and Mateus, 2001).

The biological activity of tannins consumed by animals depends not only on their content and structure, but also on the physiological status of the animals. The quantity of nutrients in the diet (*i.e*., protein) of the animals varies according to their physiological capacities and needs, which thereby affect the biological activity of the tannins. These factors should be taken into account when drawing conclusions on the effect of tannins (Robbins *et al*., 1991, Waghorn, 2008).

3.2.3 Prodelphinidin (PD):Procyanidin (PC) ratio

A higher PD:PC ratio enhances the ability of CT to complex with proteins (Aerts *et al*., 1999). Guimaraes-Beelen *et al*. (2006) observed a small variation of the PD:PC ratio between the phenological stages (full growth and flowering) on *Mimosa hostilis*. However, there was a higher PD:PC ratio on *Mimosa caesalpinifolia* and *Bauhinia cheilantha* at the stage of fructification. According to Marais *et al*. (2000), PD:PC ratios in different sainfoin varieties vary from 36:64 to 93:7.

Polymer composition affects CT properties (Sarni-Machado *et al*., 1999; Frazier *et al*., 2010) and is also thought to affect how CT influences digestion and nutritive value of the forages (Meagher *et al*., 2004; Mueller-Harvey, 2006). *Lotus corniculatus* and *Lotus pedunculatus* differ in their CT contents (Terrill *et al*., 1992a) and in their chemical structures (Meagher *et al*., 2004). The PD:PC ratio was 16:84 for *Lotus corniculatus* and 70:30 for *Lotus pedunculatus* (Foo *et al*., 1982; Hedqvist *et al*., 2000; Min *et al*., 2003b). Therefore, sainfoin CT structures appear to resemble *Lotus pedunculatus* CT (Foo *et al*., 1982; Marais *et al*., 2000), but the nutritive value of sainfoin is reportedly much higher than *Lotus pedunculatus*, as reviewed by Min *et al*. (2003b). This suggests that the relevant differences in CT structure between sainfoin and *Lotus pedunculatus* have not yet been identified.

3.2.4 Stereochemistry

The stereochemistry of the heterocyclic C-rings can take the form 2,3-*cis* or 2,3-*trans*, and this dictates how the monomeric units are attached relative to one another. The *cis:trans* stereochemistry of the monomeric subunits could affect CT interaction with proteins. However, the literature contains very little information on this topic (De Freitas and Mateus, 2001). There is broad variation in the stereochemistry of CT among cultivars and growth stage.

Koupai-Abyazani *et al*. (1993a) found that the *cis*:*trans* ratio in sainfoin decreased from 84:16 in the youngest, still unfolded leaves at the top of the stem, to 48:52 in slightly older, more mature leaves at the bottom of the stem. Marais *et al*. (2000) confirmed that sainfoin tannins appear to have a highly variable composition, with *cis:trans* ratios ranging from 47:53 to 90:10.

According to Barry and McNabb (1999) it is still difficult to link all the parameters that characterise CT with their effects on forage digestion. For example, as the previous authors suggested, protein precipitation by CT may be more responsive to the mDP and less affected by the PD content. They also suggest that the rate of degradation of protein by rumen microbes may be more responsive to differences in CT structure than in protein–binding activity.

3.3 Occurrence, location and role of tannins in plant tissues

CT are widely distributed throughout the plant kingdom, primarily in dicotyledonous species (Waghorn *et al*., 1977; Frutos *et al*., 2004; Larkin *et al*., 1997). Tannins are often present in

trees, shrubs and herbaceous leguminous plants (Perevolotsky 1994). Some such leguminous species are sainfoin (*Onobrychis viciifolia*), sulla (*Hedysarium coronarium*) and *Lotus* (*pedunculatu*s or *corniculatus*). Tannins also occur in species of Pine *(Pinus)*, wattle *(Acacia)*, oak *(Quercus)* and apple, strawberry *(Rosaceae)* (Ben Salem 1998).

CT are secondary metabolites produced by plants for which no role has yet been found in growth, photosynthesis, reproduction, or other "primary" functions (Mueller-Harvey and McAllan 1992). The CTs are located mainly in the cell vacuoles and their pattern of distribution is free-unbound, protein-bound or fibre-bound. In these sites they do not interfere with plant metabolism. Only after cell breakdown and death can they act and produce metabolic effects (Terrill *et al*., 1992b; Frutos *et al*., 2002).

Both classes of tannins, HT and CT, can be found in the same plant species, but their distribution can be different among the organs of the plant (Jean-Blain, 1998). Locations of tannins in various plant tissues comprise:

- Bud tissues: most common in the outer part of the bud, probably as protection against freezing (Luthar, 1992)
- Leaf tissues: most common in the upper epidermis. However, in evergreen plants, tannins are evenly distributed in all leaf tissues. They serve to reduce palatability and thus protect the plant against predators (Brownlee *et al*., 1990).
- Root tissues: most common in the hypodermis (just below the suberized epidermis). They probably act as a chemical barrier to penetration and colonization of roots by plant pathogens (Safdar Ali Kayani *et al.,* 2007).
- Seed tissues: located mainly in a layer between the outer integument and the aleurone layer. They have been associated with the maintainance of plant dormancy, and have allelopathic and bactericidal properties (Scalber, 1991).
- Stem tissues: often found in the active growth areas of the trees, such as the secondary phloem and xylem and the layer between epidermis and cortex. Tannins may play a role in the growth regulation of these tissues. They are also found in the heartwood of conifers wherer they may contribute to the natural durability of the wood by inhibiting microbial activity (Waterman and Mole 1994).

According to Bell *et al*., 1992, the highest contents of tannin (15-20 %DM) occur in young leaves, bracts, carpel walls and seedcoats of fruiting plants. Older leaves and bracts, terminals, squares, young bolls and root and stem bark generally contain 5-15 % tannin.

3.4 Biosynthesis of condensed tannins

The first steps in the biosynthesis of flavan-3-ols take place in the cell cytoplasm from phenylalanine and acetate precursors (**Figure 6**), whereas the maturation and the polymerisation of the CT happen in the cell vacuoles of plants (Aerts *et al*., 1999; Waghorn,

2008). Flavonoid biosynthesis is a variable character that may be under different control, in different parts of the same plant (Foo *et al*., 1982). For example, proanthocyanidin polymers isolated from leaves and roots of *Lotus corniculatus* vary considerably in their PD:PC ratio, probably owing to a variation in the biosynthesis in different parts of the plant.

Two independent processes of tannin biosynthesis have been identified in mutants of *Lotus peduncularus*: a) light-mediated and occurring in the apical meristerm and b) nutritive and occurring in the root system. Biosynthesis of CT in leaves was controlled by light quality and in the roots by stressing the plants in nitrogen deficiency conditions.

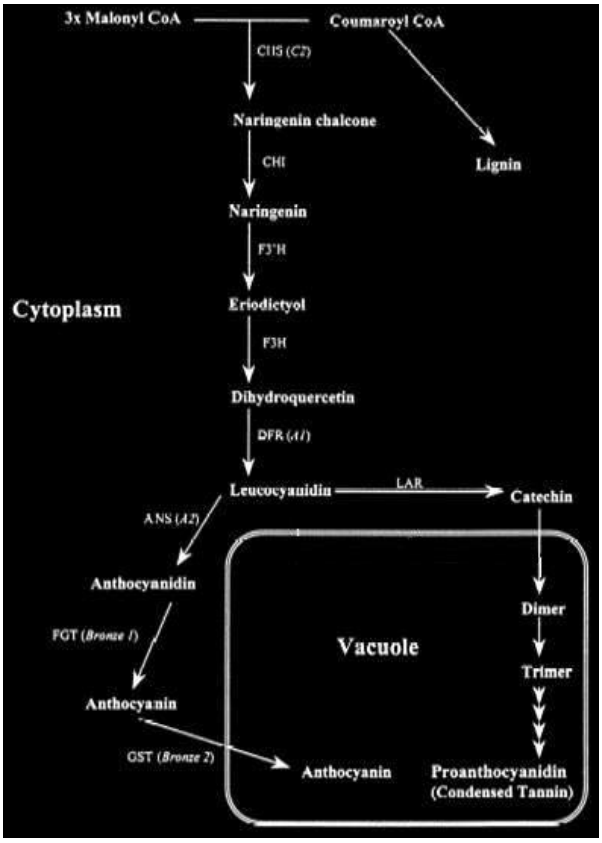


Figure 6: Pathways of proanthocyanidin and anthocyanidin biosynthesis (Aerts, 1999).

3.5 Factors affecting the biosynthesis of condensed tannins

3.5.1 Related to plant

- **Genus, species, variety of plant**

CT contents and distribution varies among species and cultivars (Kelman and Tanner 1990; Roberts *et al*., 1993). As already mentioned, CT occur in both dicotyledonous and leguminous species. However, even in the same botanical family the content of CT varies (Anuraga *et al.*,

1993). For example, Hoskin *et al*., 2000 found that sulla (*Hedysarium coronarium*) contained 35 gCT/kg DM and *Lotus corniculatus* 20 gCT/kg DM.

However, even within the same plant genus, differences in CT can be observed between species. It was found that CT content of *Lotus corniculatus* (Foo *et al*., 1997) was 77 gCT/kg DM when *Lotus pedunculatus* contained 48 gCT/kg DM (Foo *et al*., 1996). Acuna *et al*., 2008 mentioned that the CT content of *L. tenuis* varied from 0.7-1.6 gCT/kg DM when *Lotus pedunculatus* varied from 25-107 gCT/kg DM.

Finally, variations can be found among different varieties of the same species, such as for *Lotus pedunculatus* varieties (Anuraga *et al*., 1993) or *Lotus corniculatus* (Hedqvist *et al*., 2000).

- **Age and maturity of the plant**

The content and composition of CT changes with leaf age (Koupai-Abyazani *et al*., 1993b) and ability to bind protein tends to be higher in less mature forage plants (Bate-Smith 1973). Borreani *et al*., 2003 found in sainfoin that CT content decreased from 27 to 16 g/kg DM over the whole growth cycle. Also, during plant tissue maturation CT (proanthocyanidins (PA) can change in structure, for instance in the degree of polymerisation (Hagerman and Butler 1991; Koupai-Abyazani *et al*., 1993b). CT content, degree of polymerisation and astringency increased with maturation of leaves of four oak species at different stages of maturation (4 days old to 1 year old) (Makkar *et al*., 1991).

Bell *et al*., 1992 reported that the content of CT increased progressively in each new leaf of cotton (*Gossypium* spp.) until plateau contents were reached in leaves developed during early fruiting. They explained that once leaves are fully expanded, there is a little further synthesis of tannin. Tannin contents in root and stem bark also increase progressively with age from non detectable levels in germinating seed to more than 10% in fruiting plants.

Large quantities of catechin were formed in the early fruit in apples (*Malus domestica*), but after a few weeks fell sharply to reach a steadier level at plant maturity (Mosel and Hagerman 1974). The CT content of seeds and young seedlings of *Struthanthus vulgaris,* are low, increasing apparently exponentially with development of the seedlings, i.e. a 16-fold increase from the age of 10 to 30 days (Salatino *et al*., 1993).

According to Iason *et al*., 1993, seasonal variation (which correlates with phenological stage) results from different demand for nutrients. During their growth period when plants produce a lot of biomass, few resources are available for the synthesis of phenolic compounds. However, during flowering, when growth is reduced, excess carbon may become available for tannin synthesis.

- **Part of the plant**

CT may be found in different parts of the plant (Seigler 1995), but higher CT content mainly occurs in the leaves and flowers or fruits of the plant (Lees *et al*., 1995; Gebrehiwot *et al*., 2002; Frutos *et al*., 2002).

Iason *et al*., 2005 in *Holcus lanatus*, a temperate grass, found that floral tissue contained more CT than nonreproductive tissue.

3.5.2 Related to environment

- **Stress**

Moisture stress induced proportionately larger reductions in growth rates of big trefoil as temperature increased, and correspondingly larger rises in CT contents (Anuraga *et al*., 1993). Also, the pattern of distribution of free-unbound, fibre-bound or protein-bound CT depends on climatic or nutritive stress during the growth of the plant. Iason *et al*., 1995 observed a decreasing proportion of free-unbound CT and greater preponderance of protein-bound and fibre-bound CT with increasing leaf age and in dead matter of *Holcus lanatus*, a temperate grass.

Feucht *et al*., 1994, found that yellowing leaves of young understory beech trees contained more flavanols than green leaves. It has been suggested that flavanols are involved in defence mechanisms against environmental stress; as the uppermost crowns of tall beech trees are exposed to more sunlight and air pollution than understory trees, they may divert more carbon toward flavanol synthesis than understory trees.

For both tropical and temperate legumes, changes in CT accumulation as a result of environmental factors are evident only when the stress imposed affects plant growth. For example, a study of the tropical legume *D. ovalifolium* in areas with high rainfall through the year indicated a marked effect of soil physical properties on CT accumulation. In this case lack of oxygen in the soil (poor internal drainage associated with poor root development) affected uptake of nutrients and plant development (Lascano *et al*., 2001).

- **Light intensity**

There is a well-established positive relationship between the intensity of solar radiation and the quantity of phenolics produced by plants. Generally there is a rise in total phenolics in plants grown in sunny situations relative to shady ones. Thus more phenolics are found in shoots, exposed to greater amounts of light, than roots (Mole and Waterman, 1988)

- **Climate**

Lees *et al.* (1994) reported that *Lotus pedunculatus* Cav. (syn. *L. uliginosus*) reached higher levels of CT when it grew at 30°C compared with 20°C. Anuraga *et al*., 1993 observed that CT levels in *Lotus pedunculatus*, increased at high temperatures (32-28°C) but had only a small effect on CT levels in *Lotus corniculatus*. This means that the accumulation of CTs in temperate legumes in response to temperature is probably variable.

- **Fertility of the soil and season**

Significantly higher levels of CT were observed in plants growing in acidic soils with low fertility than in plants cultivated in soils with high fertility (Kelman and Tanner 1990).

Field studies showed that CT levels in *Lotus pedunculatus* declined significantly when the legume was grown in a high-fertility soil (Barry and Forss 1983). Soil fertility or fertilizer application has a minimal effect on CT content in legumes that have inherently low levels of CT. Fertllisation affects not only the CT content, but also the monomeric composition of CT (Lascano *et al*., 2001).

3.6 Toxicity of condensed tannins

Unlike hydrolysable tannins, CTs do not seem to cause toxic effects after ingestion, possibly owing to their more stable CT (Jasman and Longstaff 1993). It has long been known that the toxicity of tannins is related to their molecular size, since tannins with high molecular weights cannot be absorbed. This might explain the high toxicity in rats of low molecular weight tannins from *Quercus* species, while the larger, high molecular weight tannins of *Lespedeza cuneata* seem not to be toxic (Frutos *et al*., 2004). Ruminants are more tolerant than monogastric animals to plants rich in tannins. Their tolerance may be due in part to the microbial ecosystem responsible for fibre degradation, and which appears to modify the CT polymer before it reaches the intestine (Waghorn *et al*., 1999). The consumption of tropical tree legumes with high CT content (species of *Acacia*) is associated with increased mortality of ruminants. These species may contain high levels of proanthocyanidins and low levels of protein. CTs are not absorbed, but may affect the mucosa of the digestive tract, which could decrease absorption of other nutrients. CT will decrease the absorption of essential amino acids, such as methionine and lysine, when present at high contents. Decreased methionine availability could increase the toxicity of other plant compounds such as cyanogenic glycosides, as methionine is involved in the detoxification of cyanide (Reed 1995).

3.7 Preparation of plant material and extraction of condensed tannins

- **Preparation of plant material**

The initial harvesting, drying, storage and extraction methods have significant effects on condensed tannin analysis (Schofield *et al*., 2001).

Condensed tannins are confined to intracellular vacuoles (Bate-Smith 1957; Terrill *et al*., 1990; McMahon *et al*., 2000) and are essentially unreactive until released by cell rupture (Waghorn *et al*., 2003). This may be why tannins in fresh samples are likely to show low complex formation and oxidative polymerisation and high solubility in aqueous organic solvents (Reed 1995).

Drying methods also play an important role in sample preparation, as tannin analysis can change the phenolic compounds to some degree, depending on the species and the maturation stage of the plant. Total condensed tannin decreased slightly with temperature under aerobic drying (Palmer *et al*., 2000; Terrill *et al*., 1990). At low temperatures (<40°C), enzymes can still function, leading to oxidation. However, at temperatures above 60°C heat damage and polymerisation may occur (Haslam 1966; Goldstein and Swain 1963).

Also, most CTs are present in cell vacuoles (Tanner *et al*., 2003; Bate-Smith, 1957) and during oven-drying these vacuoles rupture and CT bind to proteins and polysaccharides, which can cause their extractability to be reduced.

Storage of plant material is necessary. Freeze-drying is considered to be one of the safest methods for preserving tannins, because temperature, air and light exposure can reduce the intensity of the oxidation reactions of the analytical methods (Makkar *et al*., 2000; Mould and Robbins, 1981; Ferreira *et al.,* 2004). Freeze-dried plant material collected and immediately frozen in the field provides dried samples that are closer to living tissues (Terrill *et al*., 1990).

- **Extractable condensed tannins**

Condensed tannins are usually extracted from the plant material using a solvent such as 70% aqueous acetone or 50% methanol. The first is considered to be the most efficient solvent for extracting tannins (Fletcher *et al*., 1977; Makkar *et al*., 2000; Chavan *et al*., 2001) and also to be a powerful solvent for proanthocyanidin polymer extraction (Foo and Porter, 1980) and is recognised as the most efficient for extracting condensed tannins from tanniniferous forage plants (Terrill *et al.*, 1990; Hagerman 1987).

One problem regarding the use of aqueous acetone solvent is that other compounds such as soluble lipids and non-tannin pigments are extracted as well, and it has been suggested that these can interfere with colour development.

- **Bound condensed tannins**

Condensed tannins are present in the plant species in two different forms: free or bound, to protein or fibre of the plant (Bate-Smith, 1973; Schofield *et al.,* 2001). Bound tannins occur because at neutral pH, condensed tannins in forage plants form stable complexes with protein and carbohydrates and so their extractability decreases (McLeod, 1974; Terrill *et al*., 1990).

The first method for the determination of bound tannins was developed by Terrill *et al*., 1992b, who proposed the extraction of the samples with 70% aqueous acetone solvent. Free tannins are estimated with HCl-butanol, after the extraction of this fraction with diethyl ether to remove lipids and interfering pigments. The residue from the acetone extraction is treated with sodium dodecyl sulphate (SDS) and the extract obtained reacts again with HCl-butanol assay protein bound tannins. After SDS extraction the remaining residue is also treated with HCl-butanol to assay fibre-bound condensed tannins (Barahona, 1999).

3.8 Inactivation of condensed tannins

Several possible strategies have been proposed and efforts made to reduce or avoid the negative effects of tannins and improve the nutritive value of forages. This demand for detoxification (by inactivation or removal of tannins) is increasing, especially for the areas with few plant resources. In principle this approach could be applied to any feed rich in tannins. Some of these methods for alleviating the negative effects of CT are presented below:

- **Storage**

The 'chopping of leaves and then storage' has been found to be easy and practical for farmers. In this process CT inactivation may be due to oxidation of tannins by phenol oxidases present in leaves and polymerisation to higher 'inert' polymers (Makkar, 2003).

- **Treatment with tannin-binding compounds**

A promising mitigation for CT is through treating with tannin-binding agents, such as polyethylene glycol (PEG) or polyvinylpyrrolidone (PVP). These agents could be highly effective in overcoming the anti-nutritive effects of CT, leading to improved animal performance (Makkar, 2003; Mueller-Harvey, 2006). It is considered that the affinity of PVPs for tannins is lower than that of PEGs (Makkar *et al*., 1995). PEG, a non-nutritive synthetic polymer, has a high affinity to CT and makes tannins inert by forming tannin-PEG complexes (Rogostic *et al*., 2008; Makkar *et al*., 1995; Provenza *et al*., 2000) and inactivating them through the entire digestive tract (Waghorn and Shelton 1997, Barry *et al*., 2001, Makkar, 2003). PEG can also release protein from the pre-formed tannin-protein complexes (Barry and Manley, 1986), or prevent the formation of such complexes. The dispensing of PEG to animals that have consumed tanniniferous plants can be done either as free choice in drinking water, given once or twice daily as an oral drench (Makkar, 2003; Waghorn, 2008), by mixing into a small amount of concentrate (Ben Salem *et al*., 2002), in feedblocks (Ben Salem and Nefzaoui, 2003) or by spraying on tannin-rich feedstuffs (Barry *et al*., 1986).

PEG, the tannin fraction with the most hydrophobic character forms the strongest complex, which can be attributed to direct hydrophobic interactions with PEG, known to exhibit relatively strong hydrophobic properties (Oh *et al*., 1980). Hydrophobic interactions for PEG with a molecular weight of 4000 inhibit complexation between condensed tannins and protein enzymes (Ravindra, 1992). Weak hydrogen bonding between oxygen of the PEG chain and the phenolic hydroxyl group on the tannin might explain the precipitation phenomena (Silanikove *et al*., 1996).

Hydrogen bonding between the PEG–CT complex was favoured with low molecular weight PEG (MW=200), whereas hydrophobic bonding between CT–PEG primarily occurred in the presence of high-MW PEG (MW=4600) (Sarathchandra and Mainwaring, 2005).

In most *in vivo* studies the molecular weight of PEG 3500 or 4000 has been used. In studies on the effect of PEG on livestock, sheep or goats have been used and the amount of PEG incorporated into the diets varied from 3 to 120 g/day (Getachew *et al*, 1998). Studies on sainfoin are those of Scharenberg *et al*., 2007: 100 g/day of PEG 4000, Bermingham *et al*., 2001: 160 g/day of PEG 3500, on sulla (*Hedysarum coronarium*) Bermingham *et al*., 2001: 120 g/day of PEG 3500 or on *Lotus pedunculatus* Barry and Manley, 1985: 75-100 g/day of PEG 3350. On the other hand, there are several studies with other tanniniferous plants that used even less PEG than 60 g/day, e.g. Ben Salem 1998: 20 g/day of PEG 4000 on *Acacia*

cyanophylla L.; Priolo *et al*., 2000: 40 g/day of PEG 4000 on carob pulp and Decandia *et al*., 2000: 50 g/day of PEG 4000 on *Pistacia lentiscus L.*

Incorporation of PEG by weight equivalent to the intake of total tannins or total phenols as tannic acid equivalent should be sufficient to inactivate tannins (Getachew *et al*., 2001).

There have been many *in vitro* studies confirming the inactivation of tannins by PEG and the increase in the nutritive value of these forages measured by the *in vitro* gas production technique (IVGPT) (Karabulut, 2006; Getachew *et al.,* 2002; Canbolat, 2005; Madibela *et al*., 2006; Rubanza *et al*., 2005).

Many papers have been published on the ability of tannins to reduce diet digestibility. The administration of PEG to the animals consuming the CT was associated with an increase in digestibility (Bermingham *et al*., 2001; Scharenberg *et al*., 2007; Barry and Manley, 1986; Barahona *et al*., 1997; Pritchard *et al*., 1992).

- **Wood ash**

The cost of PEG is likely to limit its use by farmers. Another option is the use of wood ash, easily available even in small agricultural areas, and a cheap source of alkali for the deactivation of tannin-rich feedstuffs. The high effectiveness of pine wood ash was attributed to its high levels of alkali (Waghorn, 2008) Copping and soaking leaves of acacia in solutions of wood ash can deactivate the CT and increase organic matter digestibility and nitrogen retention in sheep (Ben Salem *et al*., 2005).

- **Treatment with chemicals**

Alkaline treatments, such as with Ca $(OH)_2$ or NaOH, can be very effective in preventing the toxic or antinutritive effects of tannins (Mueller-Harvey, 2006; Makkar, 2003; Wina *et al*., 2005). The reduction in tannins due to alkali treatment results from oxidation of phenolics by oxygen at higher pH values. Also, extraction with aqueous organic solvents (30% acetone, 50% methanol, and 40% ethanol) removed about 70% tannins, and oxidising agents (potassium dichromate, potassium permanganate) removed/inactivated up to 90% of the tannins in oak leaves (Makkar and Singh, 1992; Makkar, 2003).

- **Feed mixtures**

One potential strategy for herbivores would be to mix diets. Some research has shown that mixtures produced fewer adverse effects than tanniniferous browses fed as sole feeds (Dube and Ndlovu, 1995; Patra *et al*., 2003).

3.9 Interaction of tannins with other molecules

Tannins have the ability to interact with different macromolecules and metal ions, and these interactions are to a large extent due to the properties of the phenolic nuclei in the tannin molecules (Haslam 1996). Tannins form complexes with numerous types of molecules including carbohydrates, polysaccharides, bacteria and enzymes, metal ions (iron, manganese, copper,

aluminum, calcium, etc.) and proteins (Bravo 1998; Aerts *et al*., 1999; Mueller-Harvey and Mc Allan 1992).

3.9.1 Proteins

Condensed tannin-protein interactions are the basis of many biological effects of tannins, and probably one of the best-known properties of polyphenolic compounds is their capacity to bind and precipitate protein.

The environmental conditions, under which complexations take place, and the characteristics of both tannin and protein, have been shown to cause considerable variations in the strength of this interaction.

- pH

The CT-protein complexation is pH-dependent and therefore reversible. An *in vitro* study showed that complexes between lucerne leaf RuBisCO protein and sainfoin CT were stable in the range pH 4.0-6.5, but dissociated and released protein at pH <3.0 (Jones and Mangan 1977) (**Figure 7**). The precipitation of protein by tannins is maximized at or near the isoelectric point of the protein. It is at this pH that electrostatic repulsions are minimal (preventing aggregation of protein) and allow carboxyl oxygen to bind with the tannin's hydroxyl group (Van Buren and Robinson 1969; Hagerman and Butler 1981). Interactions are dynamic and time-dependent, and the process is likened to "fitting a hand into a glove" (Haslam 1996).

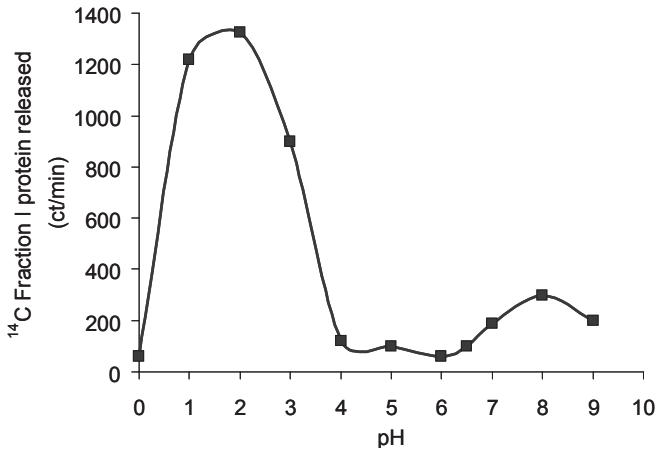


Figure 7: Effect of pH on the tannin-lucerne Fraction 1 protein complex.

- Molecular weight and structure of proteins

The extent of the association of tannins with proteins appears to be affected by the molecular weight of polymer (Porter and Woodruffe, 1984), the predominance of prodelphinidins relative to procyanidin units, the types of terminal groups and the structure of the two molecules involved (Mueller-Harvey and Mc Allan, 1992; Poncet-Legrand *et al*., 2006). Concerning proteins, those with a high molecular weight (higher than 20 kDA) and a less tightly folded

secondary and tertiary structure have a high affinity for polyphenols. Also, proteins with high contents of hydrophobic amino acids (proline) increase their capacity to form strong hydrogen bonds due to increased accessibility of the peptide linkages (Min *et al*., 2003b; Hagerman, 1992; Zimmer and Cordesse, 1996; Jean-Blain, 1998).

- Conformational flexibility

Conformational flexibility in both polyphenol and protein are important complimentary factors, leading to strong interactions (**Figure 8**). Tannins have a large number of free phenolic hydroxyl groups that form strong hydrogen bonds with proteins and carbohydrates. Tannins must be small enough to penetrate interfibrillar regions of protein molecules, but large enough to crosslink peptide chains at more than one point (Reed, 1995).

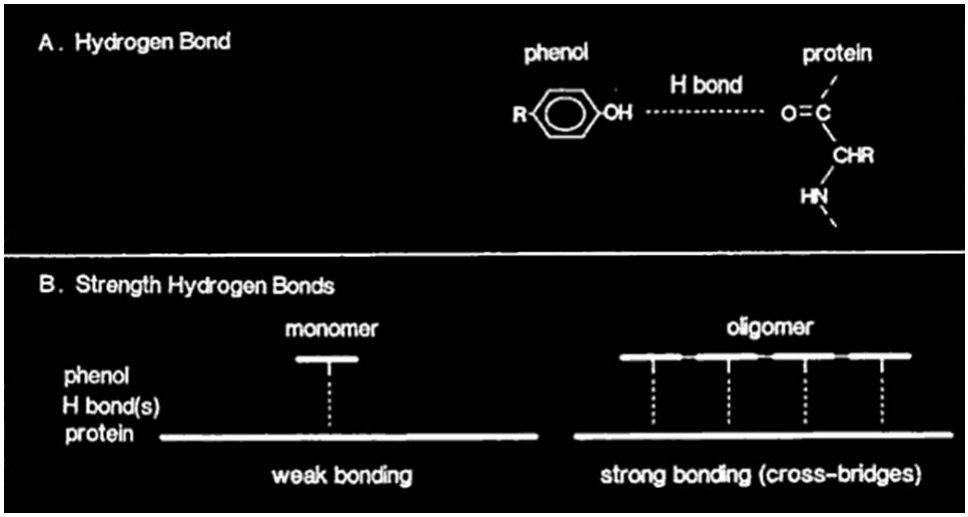


Figure 8: Hydrogen bonding of tannin compounds with proteins. A. Location of the hydrogen bond between the hydroxyl group of the phenol and the carboxyl group of the protein peptide. B. The role of the cross-bridged hydrogen bonds on the strength of the tannin-protein interaction.

- Solubility of tannin

There is a broad inverse relationship between the strength of the association with protein and the solubility of the CT in water, so low solubility favours strong association (Waterman, 1999; Spencer *et al*., 1988).

- Purification and content of protein and tannin

The purification of both tannin and protein is another parameter that influences the complexation of these molecules. It was observed that less than 15 min was needed for the reaction between pure tannin and protein, whereas more than 15 hours contact was necessary between purified casein and non-purified tannin extracted from oak leaves (Zimmer and Cordesse, 1996).

The formation of complexes between tannin and protein depends on the content of both tannin and protein. When protein contents are higher than tannin contents, the optimal ratio between tannin and protein is achieved and formation of these complexes is maximised. When the reverse is true, the polyphenols associate at one or more sites at the protein surfaces, and little precipitate is formed. Finally, a large excess of protein leads to the formation of a small precipitate (Jones and Mangan, 1977, Asquith *et al*., 1987, Mole and Waterman, 1988): at higher protein contents, protein in the precipitate is associated with relatively less and less tannin, until a point is reached where precipitates become less stable due to the smaller percentage of tannins incorporated in them.

- Ionic strength

The CT–protein complex also depends on ionic strength (Oh *et al*., 1980). The presence of Ca, Mg, Na and K is known to improve the activity of extracted leaf ribulose biphosphate carboxylase and increase the ease with which insoluble tannin-plant protein complexes are formed (Viitanen *et al*., 1990). An *in vitro* study confirmed that the presence of inorganic ions in the rumen fluid was essential for the formation of insoluble tannin-protein complexes at pH values normally found in the rumen (Perez-Maldonado *et al*., 1995).

- Temperature

The complexation of the CT molecules and protein also depend on the temperature. Hydrophobic bonding strength increases with ionic strength and temperature (Oh *et al*., 1980).

Chemical linkages

The interactions of CT with other molecules occur mainly by hydrogen bonding and hydrophobic bonding (reversible) or less frequently covalent bonding (Oh *et al*., 1980 Haslam, 1996; Frazier *et al*., 2010). It is considered that the intermolecular complexation between CT and proteins involves both hydrophobic interactions and hydrogen bonding. The first step of the CT-protein complex consists in polyphenols searching for the appropriate sites of proteins where hydrophobic bonding will be occur. Hydrophobic interactions are relatively long-range, and depend on the respective solubility of both the protein and the polyphenol moiety in the solvent. The next step is the formation of hydrogen bonds, which are considered to occur mainly between the hydrogen donors in the form of phenolic hydroxyl groups in the tannins and hydrogen acceptors in the form of carboxyl groups in the peptide linkages of the proteins. This short-range phenomenon tends to stabilize the complex formed (Poncet *et al*., 2006, 2007; Ben-Salem, 1998).

Tannins form covalent bonding under oxidative polymerisation under conditions of heating, at higher pH (pH>8), exposure to ultraviolet radiation or the action of polyphenol oxidase (Reed, 1995) or autoxidation to quinones. The quinones then react with proteins, with irreversible covalent bonding (Le Bourvellec *et al*., 2004)

3.9.2 Carbohydrates

The type of bonding between polyphenolics and carbohydrates it is not well known. Some proanthocyanidins may be covalently linked *via* ether bridges at C-4 to carbohydrates, analogously to the C-C bridges in proanthocyanidins, but the evidence for such linkages is circumstantial. Other types of bonding between polyphenolics and carbohydrates have been demonstrated; H+-bridges and hydrophobic interaction binding are important in such complexes (Mueller-Harvey *et al*., 1987).

McManus *et al.,* 1985 studied binding among several polysaccharides and polyphenolics. They concluded that where the association was primarily a surface effect, broad similarities were noted with the analogous complexation with proteins. The molecular size of the polyphenol and its flexibility were important to the binding, which, however, seems to be pH-independent. Small changes in the structure of either the polyphenol or the polysaccharide resulted in marked changes in their affinity for each other.

3.9.3 Metal ions

Tannins are also chelating agents, and this can reduce the availability of certain metal ions (Frutos *et al*., 2004). The ability of tannins to complex with metal ions is due to the ortho-hydroxyls present in their B-rings and it was found that ion exchange was the main adsorption mechanism, as metal cations displace the adjacent phenolic hydroxyl groups forming a chelate (Vázquez *et al*., 1994; Zhan and Zhao, 2003). Tannins in nature are able to react with heavy metal ions in aqueous solutions. However, their solubility in water restricts their function as metal ion adsorbents in aqueous solutions (Liao *et al*., 2004).

3.9.4 Enzymes and bacteria

The reaction between CT and bacteria cells is less well understood than reactions between CT and forage proteins. However, the CT-bacterial interaction is much stronger than the CT-protein interaction and is not reversible with PEG. CT are reactive towards the cell walls of bacteria and the extracellular enzymes secreted inhibit their activity and growth (**Figure 9**). The extent of inhibition may differ with different types and contents of CT (McSweeney *et al*., 2001). Stimulation of growth can be reversed by increasing the content of CT. At higher contents (<200 $mg \cdot mL^{-1}$ of CT), CT precipitate plant protein and therefore less are available for bacterial proteolysis. Higher contents of CT may also act by inhibiting the bacterial proteinase enzyme either directly, by inhibiting and blocking the active site, or indirectly, *via* inhibition of general bacterial metabolism (Molan *et al*., 2001). Though stronger, the CT-bacterial linkage would not be expected to predominate in the rumen. CT bind with many different kinds of compounds, ansd so CT binding in the rumen will be partially determined by the availability of these compounds in the rumen, and not solely by the strength of any particular interaction.

Also, closely related bacterial strains of the same species can differ significantly in their tolerance of tannins (McSweeney *et al*., 2001; Min *et al*., 2003b).

Rumen digestion of feed components by the microbiota (bacteria, protozoa and fungi), under anaerobic conditions, results in the production of gases (CO_2 and CH_4) eliminated through eructation. An antimethanogenic activity has been attributed mainly to the group of CT due to their interaction with rumen bacteria (Martin *et al*., 2010).

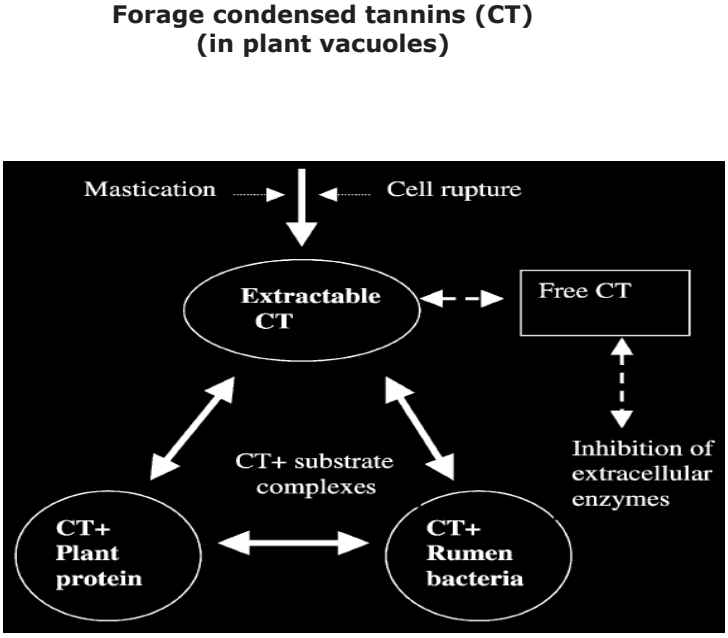


Figure 9: Effect of condensed tannins (CT) on rumen bacteria and plant protein in the rumen and their interactions (Min et al., 2003b).

3.10 Analytical methods for condensed tannins

The study of condensed tannins has been difficult because of the great variation and diversity in their structures. Nevertheless, many analytical methods have been developed based on their physicochemical or biological properties (**Table 2**).

Most of the methods for the assay of condensed tannins can be classified as colorimetric, protein precipitation or mixed assays and structural characterisation assays.

3.10.1 Colorimetric assays

- **Acid-Butanol assay**

The acid-butanol assay is the most commonly used colorimetric method for the assay of condensed tannins in plant tissues. Bate-Smith (1954) was the first to develop a technique for the detection of proanthocyanidins in plant tissues. Later many scientists, such as Porter *et al*.

(1986) developed the method, which is based on the acid-catalysed oxidative depolymerisation of condensed tannins to yield red anthocyanidins that can be detected spectrophotometrically (Schofield *et al*., 2001; Makkar *et al*., 2000).

The acid-butanol should be used with caution for the quantitative determination of tannins as tannin polymers are cleaved into dimers or trimers instead of monomers and this leads to underestimation. The acid-butanol procedure can detect very low CT levels, but appears to be most accurate for CT contents of approximately 1% DM or higher. However, this method may be useful as a screen for low tannin-containing plants, particularly if the CT is in bound forms (Terrill *et al*., 1992b).

In addition, as Terrill *et al*., 1994 confirmed, another problem with this assay is that it underestimates condensed tannin content in digesta and faecal samples, probably as a result of changes in the structure of condensed tannins during their travel through the ruminal gastrointestinal tract, reducing conversion to anthocyanidin on acid hydrolysis.

- **Vanillin assay**

This assay is specific for condensed tannin and depends on their reaction with vanillin and the formation of coloured complexes. However, this vanillin–HCL procedure is unsuitable for the assay of fibre-bound tannin, as there is no colour development with this fraction. With extractable bound CT the advantage of this assay is that it is very sensitive to low levels of CT (Terrill *et al*., 1992b). Also, although the most commonly used standard is catechin, under normal reaction conditions the catechin monomer has a different reaction rate from that of related procyanidin polymers, leading to underestimation of large polymers (Hagerman 1998).

Thus structural variations in CT will also affect the colour yield with vanillin, but as already stated the complexity and variability of the condensed tannin structures account for these analytical difficulties. Critical to the successful use of this assay could be the type of solvent used, the nature and content of the acid, the reaction time, temperature, vanillin content and type of reference standards used (Schofield *et al*., 2001).

The acid-butanol assay measures the total number of flavanoid residues present in the sample, while the vanillin assay measures the number of molecules.

- **The DMACA (4-dimethylaminocinnamaldehyde) assay**

This method is used to measure the flavan-3-ols, the monomers of CT (Li *et al*., 1996; Thies and Fischer 1971; Treutter 1989) and is more sensitive to the detection of monomers than polymers (Gutmann and Feucht 1994).

3.10.2 Protein precipitation assays

- **Radial diffusion assay**

The protein-precipitating capacity (biological activity) of the tannin can be determined by the amount of protein precipitated by extracts from tannin-containing samples. This capability is exploited in the radial diffusion assay (RDA) introduced by Hagerman 1987.

The radial diffusion assay depends on the formation of complexes between tannins and bovine serum albumin embedded in an agar solution (Hagerman 1987). Plant extracts are placed in a well in the agar and allowed to diffuse through it. As the tannin diffuses into the gel and complexes with protein, a circle of opaque complex is formed around the well. The diameter of the circle is proportional to the amount of tannins in the extract.

As in the case of all the colorimetric procedures, this method depends on the suitability of the standards. The most commonly used standard is tannic acid and the results are expressed as tannic acid equivalent. This kind of assay is suitable for screening large numbers of samples and (or) of unusual composition, and requires neither complex reagents nor instruments. It may be more appropriate for estimating astringency than for quantifying the tannin content.

3.10.3 Mixed assays

A mixed assay combines some of the preceding methods for the purpose of eliminating their major problems and reducing the time required for analysis. One assay consists of the extraction of tannins from plant samples using 70% acetone, and the analysis is performed using acid-butanol, the traditional method. The novelty of this assay is that instead of using an external standard such as quebracho (as suggested by HCl-butanol) internal standards (tannins from the same plant being analysed) are used (Giner-Chavez *et al*., 1997).

Although the use of external standards may be essential when comparing tannin contents of different plants, no single standard can be used for all plant species. The limitation of using the external samples is simply that every increment of an external sample has a different absorption from every increment of the tannin from the plant extract. In many cases, it is advisable to use a purified standard from the plant of interest (Hagerman and Butler 1991). Giner-Chavez *et al.,* 1997 found that the tannin fractions were underestimated when cyanidin or delphinidin were used as external standards and overestimated when purified quebracho was used. Hence the use of internal standards provides a more realist estimation.

3.10.4 Structural characterisation assays (acid cleavage reactions)

The acid cleavage reactions include the acid-catalysed condensation reactions with benzyl mercaptan and phloroglycinol (Schofield *et al*., 2001).

- **Thiolysis**

The quantitative and also qualitative analysis of CT in a sample can be performed by the thiolysis method, which is based on the cleavage of the CT interflavan links (Guyot *et al*., 2001; Gu *et al*., 2003; Gea *et al.,* 2011). These reactions result in the release of the chain ending unit at the base, as free-3-ols, whereas all internal units appear as benzyl thioethers. This release

permits the determination of both chain length and composition of tannins when the thiolysis products are analysed by HPLC (Schofield *et al*., 2001).

Quantitative data give the content of extractable plus unextractable CT; qualitative data provide information on tannin structures, *e.g.*, mean degree of polymerisation (mDP) *syn.* molecular size, prodelphinidin:procyanidin (PD:PC) and *cis*:*trans* ratios (Schofield *et al*., 2001; Meagher *et al*., 2004).

The HCl-Butanol assay permits the determination of the total quantity of tannins in the extract of the plant, whereas with the thiolysis method both the percentage of each monomer in the tannin and the mean degree of polymerisation can be determined.

Two advantages of this method are that it is possible to calculate the degree of polymerisation and that, unlike HCl-Butanol hydrolysis, it is less dependent on many factors.
It is thus a method of choice when coupled with HPLC-UV/ESI-MS analysis as it also possible to determine the quantity and nature of each chain-end monomer.

Table 2: A summary of available assay methods for condensed tannins (CT)

Assay name	*Assay type*	*Strengths*	*Weaknesses*	*Comments*
Acid-butanol	Chemical, Colorimetric	Specific for CT Simple, quick	Requires external standard. Colour yield varies with the tannin structure. Tannin polymers are cleaved into dimers/trimers instead of monomers, and this leads to underestimation.	Based on oxidative depolymerisation of CT in butanol-HCl reagent. The acid-butanol assay measures the total number of flavanoid residues present.
Vanillin	Chemical, Colorimetric	Used for CT but is not so specific	Measures CT as simple flavonoids, catechin the commonly used standard has a different reaction rate from procyanidin polymers, low molecular weight flavanols overreact and large polymers underreact.	The vanillin assay measures the number of molecules of flavonoids. It measure CT as well as simple floavonoids. Unsuitable for determination of fibre-bound tannins.
DMACA	Colorimetric	More sensitive than vanillin-HCl		Permits the assay of flavan-3-ols, the monomers of CT. More sensitive for monomers than polymers.
Mixed assays	Mixed	Specific for CT, instead of external standard internal are used, less time consumed.		
Thiolysis	Chemical, needs HPLC	Good for structure determination	Requires pure tannin Problems with heterogeneous material (e.g. grassland)	Benzyl mercaptan is unpleasant to handle
Radial Diffusion	Protein precipitation	Determination of large numbers of samples with limited laboratory facilities	Less useful for quantification than the colorimetric procedure	The most commonly used standard is tannic acid and the results expressed as tannic acid equivalent Both condensed and hydrolysable tannins can be detected

4. Nutritive effects of condensed tannins

4.1 Voluntary feed intake and palatability

In general, there is an inverse relationship between tannin contents in browse sources and voluntary feed intake by herbivores (Silanikove *et al*., 2001).

Waghorn *et al*., 1999 in his review state that levels of between 50-120 gCT/kg DM decreases voluntary forage intake, whereas other studies have reported that tannins do not influence feed intake.

Alonso-Diaz *et al*., 2009, feeding fresh foliage of tanniniferous trees (*Lysiloma latisiliquum*, *Acacia pennatula* and *Piscidia piscipula* and *Brosimum alicastrum*), concluded that tannins do not affect the intake of tanniniferous plants by sheep with browsing experience. However, sheep increased their selectivity when only tanniniferous plants were offered. Nunez-Hernandez *et al*., 1991 found that PEG treatment had no effect on *Cercocarpus montanus* intake by sheep. Bhatta *et al*., 2000 found that the presence of tamarind (*Tamarindus indica)* seed husk in the concentrate mixture of crossbred lactating cows did not affect the dry matter intake compared with the same mixture without addition of *Tamarindus indica*. The voluntary feed intake of sheep fed a mixture of grass and *Lotus corniculatus* with the addition of PEG was not different from another group of sheep fed without offered PEG (Waghorn *et al*., 1997). There was no difference between the CT acting group (ewes grazing *Lotus corniculatus)* and the PEG supplement group (ewes grazing *Lotus corniculatus* and offered daily PEG) (Min *et al*., 2001).

On the other hand, Waghorn *et al*., 1995 found that sheep fed a fresh mixture of *Lotus pedunculatus* and ryegrass ate 5-6% more dry matter that sheep fed the same diet drenched daily with PEG. Another study that gave sheep a greater opportunity for diet selection (*ad libitum*), showed a 20% increase in voluntary intake when extractable CT from *D. ovalifolium* were reduced with PEG (Carulla, 2001).

Ben-Salem *et al*., 2005 feeding *acacia* or oaten hay to lambs, observed a low intake of acacia compared with hay. This could be the consequence of astringency caused by the high tannin content in acacia. Douglas *et al*., 1999 observed that at *ad libitum* allowances, the voluntary feed intake of sheep receiving PEG appeared to be substantially reduced compared with lambs not receiving PEG (*L. cornicularus* by 27% and 35% with sulla).

Intake of *D. ovalifolium* and *F. macrophylla* increased by an average of 10% when extractable CT were reduced from 90 to 50 g/kg of DM with PEG. Although leaves contained about 2.5 times more tannins than stems, sheep preferred leaves of *D. ovalifolium* over stems. Studies have shown that leaves in tropical forages are more readily consumed than stems, because of the lower resistance of the leaf to chewing and longer ruminal retention time of the more fibrous stem particles (Barahona *et al*., 1997).

Silanikove *et al*., 1996, reported that tannin levels of 20%DM drastically reduced *Pistacia lentiscus* leaf intake by goats. Henkin *et al*., 2009 reported that in the case of *P. lentiscus*, 65%

of the available green material was consumed by the cattle group that had received PEG and only about 49% in the control group (without PEG).

Some mechanisms have been suggested to explain the negative effects of high CT contents on voluntary feed intake:

- Reduced feed palatability.

Tannins cause an astringent sensation in the mouth of the animal. This sensation results in negative feedback, which induces the animal to reduce consumption of tannin-rich feeds (Provenza and Roop, 2001). A reduction in palatability could be caused through a reaction between the tannins and the salivary and mucous proteins or through a direct reaction with the taste receptors, casuing an astringent sensation (Frutos *et al*., 2000).

- Slowing of digestion.

Reduction in the rate of evacuation of digesta from the rumen causes a higher gut-fill effect. (Baumont *et al*, 2000).

- Decrease in microbial activity

After dilution and mixing of ingested matter (containing tannins) in the ruminal fluid the activity of microbes decreased, resulting in decreased digestibility (Landau *et al*., 2000).

- Emetic effect

For grazing and more for browsing ruminants, numerous plants contain secondary metabolites that have an emetic effect and so reduce voluntary intake (Decruyenaere *et al*., 2009).

- Influence on hormones

Tannins may have an effect on hormones or metabolites responsible for intake regulation (Barry, 1984).

Waghorn *et al*., 1994, suggested that decreased ruminal turnover and rate of digestion was more important than palatability in reducing intake by sheep fed pure diets of *Lotus pedunculatus* in comparison with sheep fed *Lotus pedunculatus* along with PEG.

4.2 Nitrogen metabolism

4.2.1 Effect of condensed tannins on ruminal protein degradation

Proteins in forage legumes are often poorly utilised by ruminants because of their extensive, rapid degradation in the rumen (Broderick, 1995; Min *et al*., 2003b; Gebrehiwot *et al*., 2002). In the rumen, up to 70% of soluble forage proteins may be degraded, yielding ammonia as a by-product of amino acid deamination (McMahon *et al*., 2000).

The presence of CT increases the efficiency of protein utilisation in ruminants by protecting protein from rapid microbial degradation in the rumen (McMahon *et al*., 2000; Waghorn, 2008) (**Table 3**)**.** This effect of CT on protein degradation is basically a reduction in the immediately degradable fraction and a reduction of the fractional rate of degradation (Frutos *et al*., 2004).

The amount of plant protein surviving in the abomasum is called *by-pass protein* (Larkin *et al*., 1997). The digestion of forage protein in the rumen can be attributed to the combined processes of solubilization and degradation. Several authors reported reduced rumen ammonia contents in response to tannin-containing plants. This has been attributed to lower solubility and reduced deamination of plant proteins when tannins are present (Aerts *et al*., 1999).
McNabb *et al*., 1996 investigated whether CT affected protein solubilization, protein degradation or both. The action of CT reduced RuBisCO digestion in the rumen of sheep fed on fresh *Lotus pedunculatus*, primarily owing to the ability of CT to slow its degradation by rumen micro-organisms without affecting its solubilisation.
Tanner *et al*.,1994 investigated the effect of the content of CT purified from *Onobrychis viciifolia* on the rate of hydrolysis of the large subunit and the small subunit of RuBisCO by rumen microflora *in vitro* and showed that CT from sainfoin inhibited the proteolysis of casein and of the large RuBisCO subunit.

Scharenberg *et al.*, 2009 found a decline in ruminal ammonia content at a similar intake of CP in cows fed sainfoin hay compared with grass-clover hay. Scharenberg *et al.,* 2007 observed that feeding dehydrated or ensiled sainfoin to lambs decreased both ruminal protein degradation and rumen fluid ammonia content. A decrease in soluble N and NH_3-N content in the rumen fluid of lambs fed sulla (*Hedysarum coronarium*) was observed by Douglas *et al*., 1999.

Mastication of CT-containing forage ruptures about 60% of plant cells, and the CT are able to bind with salivary and plant proteins. The pH 6-7 in the rumen allows stable complex formation between CT and proteins. Therefore, CT may exert their inhibitory effect on protein degradation in the rumen by protecting plant protein from cleavage by proteases, or by directly inhibiting the proteases themselves or by a combination of the two. Once past the rumen, pH shifts in the abomasum (pH<3.5) and the small intestine (pH>7) release protein from the complex, thus making it available for gastric and pancreatic digestion, resulting in enhanced digestion and absorption of amino acids in the small intestine (Jones and Mangan, 1977; Mueller-Harvey, 2006) (**Figure 10**).

4.2.2 Effect of condensed tannins on N absorption and amino acid supply to small intestine

It has been previously stated that moderate amounts of CT increase the amount of dietary protein that reaches the duodenum of ruminants. However, it can be beneficial to protein metabolism in ruminants only if it is associated with an increased absorption of amino acids in the small intestine (Aerts *et al*., 1999; Barry and McNabb, 1999). Several studies report negative effects of tannins on nutrient absorption from the small intestine (Frutos *et al*., 2004) and this could be due to (i) the persistence in the intestine of tannin-protein complexes,

which failed to dissociate in the abomasum, or (ii) tannins complexing with digestive enzymes or endogenous protein (Kariuki and Norton, 2008) or (iii) tannins once again binding to dietary proteins in the intestine or (iv) a change in intestinal nutrient absorption caused by tannins interacting with intestinal mucosa (**Table 4**).

Effects of CT on the absorption of essential amino acids from the small intestine are different in *Lotus corniculatus* and *Lotus pedunculatus. In vivo* measurements showed that the action of CT in *Lotus pedunculatus* increased abomasal flow in sheep but this was counteracted by markedly reduced apparent digestibility in the small intestine (Waghorn *et al.,* 1994; Min *et al.,* 2003b).
By contrast, *Lotus corniculatus* fed to sheep increased both protein flows to the intestine and the net absorption of essential amino acids from the small intestine with no effect on apparent digestibility in the small intestine. It was found that in the presence of 23 gCT/kg DM, 50% more EAA and 14% more non-essential amino acids reached the abomasum (Waghorn *et al*., 1987a). Differing reactivities of CT with plant proteins in these two species may be due to different CT contents or CT structures.
Bermingham *et al*., 2001 found that when sheep were fed sulla (*Hedysarum coronarium*) amino acid absorption from the small intestine was markedly increased by the presence of CT. Barry and McNabb 1999 reported in their review that digestion of both fresh and dried sainfoin produced greater duodenal non-ammonia nitrogen (NAN) flow and increased amino acid absorption from the small intestine relative to digestion of fresh white clover and dried lucerne, due to the presence of CT in sainfoin.
McNabb *et al*., 1993 conclude that CT in *Lotus pedunculatus* fed to sheep resulted in an apparent absorption of methionine from the small intestine 27 % higher than sheep that received PEG.
Carulla 1994 found that the presence of CT in *Desmodium ovalifolium* increased the nitrogen (N) flux to the duodenum and the N absorption of sheep more than in those fed a diet with added PEG.
Perez-Maldonado and Norton 1996, observed higher proportions of dietary N apparently reaching the abomasum of animals given diets containing *Desmodium* (50%*)* and *Calliandra* (56 %) compared with animals given *Centrosema* and *Pangola* CT-free diets (35%).
Barahona *et al*., 1997 studied the effects of CT tropical legumes (*Desmodium ovalifolium* and *Flemingia macrophylla)* on nitrogen (N) utilisation by sheep, and found that in both legumes, the reduction of extractable CT with PEG did not improve N disappearance postruminally (even though almost twice as much protein-bound CT reached the duodenum) in sheep fed forages compared with sheep fed forages treated with PEG.

4.2.3 Effects of condensed tannins on urea cycle

The inefficient digestion of forages in ruminants results in large ammonia losses from the rumen (20-35 % of dietary N) most of which is excreted as urea in urine (Mueller-Harvey, 2006) (**Figure 10**). Condensed tannins may increase the efficiency of urea recycled to the rumen because of the lower rate of protein degradation and deamination in the rumen, resulting in lower ruminal NH_3 concentration. Sheep fed pure diets of *Lotus pedunculatus* had lower plasma urea contents, a more rapid plasma urea turnover rate and a higher irreversible loss than sheep receiving *Lotus pedunculatus* treated with PEG to "deactivate" the CT (Waghorn *et al*., 1994a).

Egan and Ulyatt, 1980 found in sheep fed ryegrass, white clover or sainfoin that the size of the urea pool, the plasma urea concentration and the irreversible urea loss did not differ between diets at similar N intake. However, for late maturity sainfoin diet, the urea irreversible loss was more than double the urinary urea output.

Min *et al*., 2001 conducted a study in which ewes grazed *Lotus corniculatus* or pasture (white clover, perennial ryegrass). They found that rumen ammonia and blood plasma urea concentration were lower for ewes that grazed *Lotus corniculatus* than for those that grazed pasture, and were generally lower for active CT than for PEG sheep grazing lotus.

Scharenberg *et al*., 2007 found that lambs fed with PEG-treated sainfoin increased plasma urea concentration compared to those fed sainfoin without PEG treatment.

In sheep fed alfalfa hay with and without tannic acid at different concentrations (0, 30, 60 and 90 g/kg DM) the blood urea-N decreased as the level of tannic acid in the diet increased (Getachew *et al*., 2008).

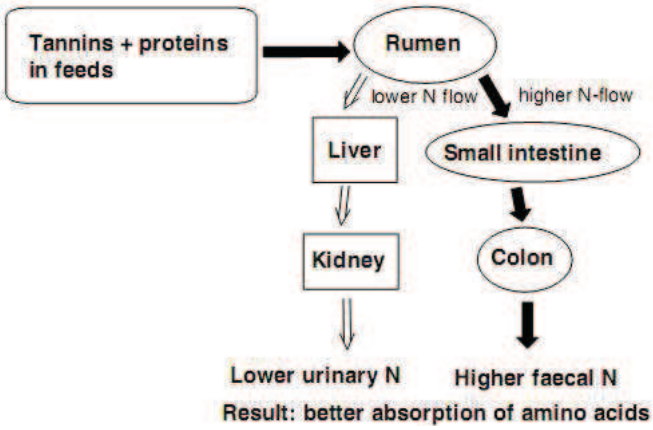


Figure 10: Condensed tannins and improvement of nitrogen utilisation (McMahon et al., 2000).

4.2.4 Effects of condensed tannins on nitrogen excretion and retention

It has been demonstrated in many studies that in animals consuming tanniniferous plants there is a shift N excretion from urine to faeces (Hervas *et al*., 2004; Waghorn, 2008)

and from soluble to insoluble N forms in faeces. Feeding CT-containing forages results in increased faecal N excretion, suggesting that this is more likely to result from increased endogenous N excretion than from the passage of indigestible CT–plant protein complexes (Kariouki and Norton, 2008; Reed, 1995).

Related to the body nitrogen retention by the animals, some studies have reported an increase, whereas others state that the nitrogen balance of ruminants is not affected by the presence of CT in forages. The improvement of N retention could be explained by the decrease of nitrogen in the urine, indicating a better use of absorbed nitrogen.

Waghorn and Shelton (1995) found that with a mixture of *Lotus pedunculatus* and *Lolium perenne*, CT did not alter N retention. Carulla *et al*., 2005 studied the effect of adding CT extract of *Acacia mearnsii* to diets containing ryegrass, ryegrass and alfalfa or ryegrass and red clover. They reported that the higher faecal N loss, caused by CT supplementation, was completely compensated for by a reduction in urinary N, without affected body N retention. According to the authors, the lack of effect on N retention is explained by decreased rumen microbial synthesis. A substantial shift occurred in the pattern of N excretion in sheep fed the tannin *versus* control silages. As a proportion of daily N intake, urinary N losses were lower and faecal N losses were higher for sheep fed the tannin silages than for those fed the control grass silage. However, there was no overall effect of tannins on N retention in mature sheep (Deaville *et al*., 2010)

Waghorn *et al*., 1987a, suggested that on sheep fed fresh *Lotus corniculatus* an increased absorption of essential amino acid could explain the high nitrogen retention.

Barry *et al*., 1986, observed that a high CT content 95 g/kg DM in *Lotus pedunculatus* increased faecal N excretion and decreased urinary nitrogen excretion, leading to higher overall nitrogen retention by sheep. The main reason suggested was the increased efficiency of use of apparently absorbed nitrogen.

The effect of supplying increasing amounts of PEG in the diet of sheep fed *Acacia cyanophilla* was studied by Ben Salem *et al*., 2000. They reported that the higher the rate of PEG introduced in feed blocks the greater the N retention.

Kronberg, 2006 found that when wether sheep fed alfalfa pellets and given tap water with 0.5, 1.0 or 1.5% of daily dry matter intake amounts of quebracho tannin, resulted in reductions in daily urea excretion.

Wiegand *et al*., 1995 compared the use of leaves from accessions of *S. sesban* as protein supplements for growing rams. They observed that faecal nitrogen was highest in rams fed the highest CT forage, whereas urinary nitrogen excretion was highest in those fed the lowest CT forage. The retention of N was maximised with the forage of intermediate CT content.

When *Desmodium intorturn, Calliandra calothyrsus* and CT-free *Centrusema pubescens* were fed to sheep and goats, it was found that higher proportions of dietary N apparently reached the abomasum of animals given the diets containing *desmodium* and *calliandra* compared with

animals given the *centrusema* diet. Sheep and goats given the CT diets also had higher excretions of faecal N. This increase in faecal N excretion was the result of an increased total flow of N and was not due to a decreased digestion and absorption of these proteins in the lower digestive tract (Perez Maldonado and Norton**,** 1996).

4.3 Effects of condensed tannins on ruminal fermentation

Among the most widely accepted mechanisms by which CT reduce the ruminal degradation of different dietary compounds are substrate inhibition, enzyme inhibition and direct action on ruminal microorganisms.

Related to the first mechanism, CT prevent or interfere with the attachment of rumen microorganisms to plant cell walls (essential for degradation). The formation of complexes with proteins or carbohydrates makes these nutrients inaccessible to microorganisms.

Concerning enzyme inhibition, it is known that CT inhibit several digestive enzymes, including proteases, pectinases, amylases, cellulases, and lipases. McSweeney *et al*., 2001 in their review state that CT from pasture legume *Lespedeza cuneata* has been demonstrated to be inhibitory to pectinase and cellulase in rumen fluid. Enzyme inhibition is believed to be caused mainly by nonspecific binding of tannins with the enzyme protein, but may also occur when tannins bind with the substrate. Many factors may influence the extent of digestive enzyme inhibition by CT, including (i) amount of protein in the diet, (ii) relative amounts of various enzymes in the diet and the order in which they are encountered, (iii) formation of tannin-protein complexes before and after ingestion, and (iv) how various enzymes are affected by pH, type of tannin, and species and age of the animal.

Finally, CT may have a direct effect on ruminal microorganism. Min *et al*., 2002 reported that in sheep after transition from a ryegrass diet to a *Lotus corniculatus* diet, populations of proteolytic rumen bacteria were decreased. When PEG was infused into the rumen of sheep fed *Lotus corniculatus*, these populations were higher than in sheep fed *Lotus corniculatus* without the PEG infusion.

CT contents of 400 µg CT/ml or more reduced the growth of a range of bacterial strains from the rumen (Molan *et al*., 2001) and produced maximum inhibition of proteolysis in *in vitro* studies (Aerts *et al*., 1999)

Aerts *et al*., 1999 confirmed that the effect of CT on the degradation of protein by rumen microorganisms may be more responsive to differences in the flavan-3-ol composition of the CT, while protein precipitation by CT may be more responsive to the molecular weight of the CT and, to a lesser extent, affected by the prodelphinidin content.

Direct measurements of microbial population shifts also give an indication of the impact of CT on rumen microflora. Chiquette *et al*., (1989) found that *Lotus corniculatus* with high CT levels (32 gCT/kg DM) tended to have higher protozoa populations than a lotus diet with 12 gCT/kg DM. However, Ben Salem *et al*., 1997 observed that the protozoa population in ruminal fluid

decreased with increasing levels of acacia. Scharenberg *et al*., (2007) found that in lambs fed sainfoin protozoa count was enchanced compared with lambs fed PEG-treated sainfoin. However, Terrill *et al*., (1992), on sulla (*Hedysarium coronarium*) found CT had a depressing effect on proteolytic protozoa.

The effects of CT on protozoa populations are important for environmental emissions from ruminants, as higher protozoa numbers tend to lead to higher methane production (Iqbal *et al*., 2008). The literature on the effect of CT on the proteolytic protozoa is still inconclusive; varying conclusions on the effect of tannins on rumen protozoa have been reported in research studies.

4.4 Carbohydrate metabolism

Results from *in vivo* studies have pointed out varying responses on carbohydrate digestion due to tannin consumption. Tannins may reduce cell wall digestibility by binding bacterial enzymes and (or) forming indigestible complexes with cell wall carbohydrates (Reed, 1995).

Barry and Manley, 1984 observed that in the rumen of sheep fed high CT (106 g/kg DM), *Lotus corniculatus,* the apparent digestibility of cellulose and hemicellulose was lower than when fed forage of lower CT content. Also, Barry and Duncan, 1984 reported that sheep fed high CT, *Lotus pedunculatus,* (63 g/kg DM) displayed reductions of 5-8 % in the apparent digestibility of cellulose and hemicellulose, respectively, compared with those fed the same forage with added PEG. It is suggested that the most probable cause of this reduction is the presence of free CT. However, sheep fed sainfoin containing 60 gCT/kg DM did not show significantly impaired of in the rumen digestion of carbohydrates, although apparent digestibility of cellulose and hemicellulose was lower compared with diets of ryegrass and white clover (Ulyatt and Egan, 1979).

The differences obtained in nutritive studies between sainfoin and lotus species may be due to higher molecular weight (MW) and lower astringency of CT of sainfoin (Jones, 1976). *Lotus* species with low MW tannins are more efficient at precipitating different substances, but also inactivate enzymes involved in rumen carbohydrate digestion (Barry and Manley, 1984).

Arrigo, 2009 found that in rams fed silage of sainfoin and lucerne the cell wall content (cellulose, lignohemicellulose and fibre) was similar, although sainfoin contained CT.

Mueller-Harvey *et al*., 1998 state that cellulose digestibility of sheep fed dried sainfoin was significant higher and the extent of cellulose digestion in the rumen was not affected compared with lucerne-fed sheep. Ben-Salem *et al*., 2005 found that NDF digestibilities were significantly increased in sheep fed acacia treated with wood ash. The same was observed by Ben Salem *et al*., 2000 when tannin containing *Acacia cyanophylla* was supplied with no PEG.

Some *in vivo* studies demonstrated that different content of CT or the addition of PEG had no effect on fibre digestibility. Nuñez -Hernandez *et al*., 2001 studied the effect of mountain

mahogany (MM; *Cercocarpus rnontanus)* leaf CT on goats and lambs using PEG to deactivate the condensed tannins. They found that digestibility of NDF was greater for animals fed diets without PEG; however, ADF digestibility was similar for both diets. Waghorn *et al.,* 1987b showed that the addition of PEG did not affect NDF digestion by sheep fed *Lotus* diets. They observed similar neutral detergent fiber digestibility at 23 g/kg DM and 50 g/kg DM contents of CT *Lotus corniculatus*.

Barahona *et al.,* 1997, fed the tropical forages *Desmodium ovalifolium* and *Flemingia macrophylla* to sheep, and found that the ruminal and total tract digestibilities of organic matter, NDF, and ADF increased for both legumes with added PEG.

Also, the digestibilities of NDF and ADF were lower in the sheep fed non-PEG treated sainfoin than in those fed the grass-clover mixture. However, PEG treatment did not totally compensate for the difference from the grass-clover mixtures (Scharenberg *et al*., 2007).

Carulla *et al*., 2005 found that NDF digestibility in lambs decreased when forages (red clover (*Trifolium pratense*) or alfalfa (*Medicago sativa*) were supplemented with 0 or 41 g *Acacia mearnsii* extract.

In addition, many *in vitro* techniques have also been used to determine the effects of condensed tannins on fibre digestibility. Makkar *et al.,* 1996 studied the nutritive value of straw and foliage of *Lathyrus sativus, Vicia narbonensis, Vicia sativa* and *Vicia ervilia in vitro*. The CT content of these feeds were quite low; 1.5, 1.3, 5.1 and 3.3 g/kg DM, respectively, for straw and 1.3 and 5.1 g/kg DM for the foliage of *narbonensis* and *sativa*.

These low levels of CT did not adversely affect their nutritive value to any significant extent, the maximum decrease in organic matter digestibility being 3%.

In 72 West African fodder trees, Rittner and Reed, 1992 found that *in vitro* NDF degradability was negatively correlated with soluble CT content in the samples.

Getachew *et al*., 2008 assessed the influence of spraying different contents of tannin acid (0, 30, 60 and 90 g/kg DM) on chopped alfalfa hay *in vitro*. They found that NDF digestibility was reduced by all levels of tannin acid compared with the control.

Rubanza *et al*., 2005 studied the effect of tannins on *in vitro* organic matter digestibility by the addition of PEG. Browse tree leaves from six species of acacia: *A. angustissima*, *A. drepanolobium*, *A. nilotica*, *A. polyacantha*, *A. tortilis* and *A. senega* was used. They observed that the addition of PEG resulted in higher organic matter digestibility with variable responses among species due to their variable levels of phenolics and tannins.

4.5 Animal performance

Since condensed tannin consumption can affect voluntary feed intake and its digestive utilisation, there is likely to be an impact on the productivity of animals consuming them.

4.5.1 Wool growth and live weight gain

Clean wool is mainly protein, with high cystine content and the availability of sulphur containing amino acids has significantly affected wool production (Min *et al*., 2003b). In their review, Aerts *et al*., 1999 reported that moderate contents of CT in the legume *Lotus corniculatus* (2-4% DM) produced significant increases in wool growth (11%), live weight gain (8%), milk yield (21%) and ovulation rate (15-30%) without increasing voluntary intake, thus improving efficiency. The increase in wool growth was probably due to the increased availability of sulphur-containing amino acids, which are major precursors of wool protein. This increase in the absorption of all essential amino acids probably contributed to increases in body growth and milk secretion.

Beneficial effects of CT, especially in *Lotus corniculatus*, for wool production occur in the range 22-38 g CT kg/DM. When CT content exceeded 50 g/kg DM, the response became negative and below 22 g/kg DM the wool growth response could be variable.

This was supported by Barry (1985) who observed that by PEG administration wool growth and live weight gain increased for sheep grazing high CT *Lotus pedunculatus* (76-90 gCT/kg DM). This result may be explained by the increase in forage intake that occurred when PEG was added to high-tannin lotus. An additional explanation was given, namely that plasma growth hormone concentration was elevated in sheep given high-tannin lotus. Growth hormone is known to stimulate N retention in ruminants, and injections of growth hormone into sheep simultaneously decrease wool growth and stimulate N retention, thus diverting amino acids away from wool synthesis into body protein deposition. Terrill *et al*., 1992a observed, during the spring and early summer, that CT in sulla (*Hedysarium coronarium*) increased wool growth rate in sheep compared with sheep grazing the same pastures, but given a daily dose of PEG. Luque *et al*., 2000 found that ewes grazing on *Lotus corniculatus* produced 11% more wool than ewes grazing perennial ryegrass–white clover pasture. This was probably due to improved efficiency of feed utilisation, and higher absorption of amino acids, particularly cysteine. Similar results were obtained in Min *et al*., 1999, where ewes grazing pasture or *Lotus corniculatus* had similar voluntary feed intakes but produced more wool with longer staples and thicker fibre diameter with supplementation. The possible cause was the action of CT in increasing plasma essential amino acids and especially branched chain amino acid concentration.

On the other side, there are some reports that tanniniferous forage plant had no effect on growth rates or wool production. Waghorn and Shelton, 1995 conducted a trial where a group of sheep was fed fresh ryegrass and two other groups were given a mixture of lotus and ryegrass with one drenched with PEG. They found that consumption with or without PEG did not affect wool growth. Also, Waghorn *et al*., 1994 found that growth rate and wool production were similar for sheep on both treatments when sheep fed *Lotus pedunculatus* with the addition or not of PEG.

It is believed that the consumption of plants containing condensed tannins can influence the live weight gain of the animals (Min *et al*., 2003b; Rochfort *et al*., 2009)

Barry *et al.,* 1985 observed a significant reduction in live weight gain in lambs fed *Lotus pedunculatus.* Moderate contents of CT in the legume *Lotus corniculatus* (2-4%) produced an increased of 8% in live weight gain (Aerts *et al*., 1999). Moore *et al*., 2003 confirmed that supplementation, for beef cattle grazing dry, sparse summer pastures, of willow (*Salix sp*) containing 27 gCT/kg DM, can reduce live weight loss (−0.36 to −0.45 kg/day) under prolonged drought conditions. Priolo *et al*., 2000 found that lambs fed a tannin-containing diet of *Ceratonia siliqua* (carob) performed very poorly, with an average daily gain of only 48 g/d, whereas adding PEG to the diet increased daily gain (140 g/d). Growth rates were highest for lambs grazing lotus compared with lambs grazing red clover or ryegrass (Speijers *et al*., 2004). The effect of CT on lamb growth depends on the degree of tannin activity (Priolo and Ben Salem, 2004).

4.5.2 Milk and meat production

Concerning milk production, the action of CT in lactating ewes grazing *Lotus corniculatus* had no effect on milk secretion in early lactation, but increased secretion rates of whole milk, lactose and protein by 21, 12 and 14%, respectively, during mid and late lactation, without affecting voluntary feed intake (Wang *et al*., 1996). This increased content of protein may be explained by the greater availability of intestinal amino acids, especially methionine and lysine, which are thought to limit milk production. The greater concentration of lactose can be explained by more glucose, as glyconeogenesis mainly involves amino acids. Goats browsing a shrubland comprising lentiscus (*Pistacia lentiscus*) and *Quercus* spp., increased milk yield and milk urea with no difference in milk fat and protein percentage, when supplemented with PEG (Decandia *et al*., 2000; Pintus, 2000).

Also, when goats received sainfoin hay or the same amount of lucerne hay, no differences for milk yield, milk protein or fat contents were observed between the two groups (Hoste *et al*., 2005). Dung *et al*., 2005 conducted a study where lactating goats were fed a rice straw diet supplemented with cassava foliage hay. They found that milk yield was linearly related to cassava hay intake, and increased by 70% (from 900 to 1500 ml/day) when cassava was consumed at 25% of DM intake compared with the zero level of cassava hay. Milk fat and protein were also increased with cassava hay supplementation.

Only in the last few years have a number of studies focused on the effects of dietary tannins on the meat quality of small ruminants.

Arrigo and Dohme, 2009 performed a study where cows were supplemented daily with either dehydrated-pelleted sainfoin or dehydrated-pelleted alfalfa. In that case, milk performance did not differ between treatments. Romero *et al*., 1997 after feeding cows with a grain silage and a base diet constituted by alfalfa pasture, maize silage, found that the values of milk production

and milk fat were lower for high tannin sorgum grain (more than 0.8% equivalent tannin acid) than for low (less than 0.4% equivalent tannin acid).

Petacchi and Buccioni, 2007 observed that the presence of the chestnut tannin product in the diet fed to treated ewes had no influence on milk yield or on the concentrations of fat or lactose in milk, but had a marked positive effect on the level of milk proteins, which were augmented up to 5.39 %.

Luciano *et al*., 2009 studied the effect of CT on meat quality when lambs were fed a concentrate diet or fed the same concentrate with the addition of quebracho (*Schinopsis lorentzii*) tannins. They found that feeding tannins resulted in greater haem pigment concentrations in the *semimembranosus* muscle during refrigerated storage.

Priolo and Vasta, 2007 report that when two sorghum varieties with different contents of tannins were compared, lambs fed the strain containing the higher level of tannins showed a meat that was lighter in colour.

CT may also affect meat odour, as the ruminal biosynthesis of odour-active compounds seems to be affected by tanniniferous diets. This is the case of skatole (3-methylindole) and indole, which confer unpleasant flavour connotations to lamb meat and are synthesized by ruminal microorganisms (Priolo *et al*., 2007). Priolo *et al.,* (2005) reported that when lambs were fed tanniniferous herbage (sulla) the deposition of skatole in perirenal fat was not affected by the supplementation of PEG, and also was higher compared with a group of lambs fed a maize-based diet. Conversely, Tavendale *et al.* (2005) have shown in an *in vitro* experiment that tannins from *Dorycnium rectum* reduce the biosynthesis of skatole in sheep rumen inocula.

The presence of CT has been associated with reduced carcass fat content in lambs grazing *Lotus pedunculatus* and *H. coronarium* (Priolo and Ben Salem, 2004). A possible explanation for this reduction of fatness has been suggested to be a lower level of growth hormone in lambs when a diet of *Lotus pedunculatus* was sprayed with PEG. Growth hormone increases N retention and reduces carcass fat deposition, with an increase in fat turnover.

Some studies have investigated the effects of feeding tanniniferous feed on lamb meat fatty acid composition with a particular focus on *cis*-9 *trans*-11 conjugated linoleic acid (CLA), a fatty acid which has favorable effects on human health. Tannin-rich diets could reduce the accumulation of CLA in lamb meat through an impairment of the activity of ruminal microorganisms (Priolo and Vasta, 2007). Vasta *et al.* (2007) found that feeding lambs a diet containing tannins (2.7% on DM) from carob reduced the accumulation of CLA in intramuscular fat, compared with animals receiving the same diet, but supplemented with PEG. Also, lambs fed PEG- and maize-containing diets had higher carcass weight, carcass yield and carcass fatness than lambs given the tannin-containing diet of *Ceratonia siliqua* (carob) (Priolo *et al*., 2000). Similarly, another trial reported that when 20% carob pulp partially replaced barley the carcass yield was reduced (Priolo *el al*., 1998).

4.5.3 Animal reproductivity

Concerning the effect of CT on reproductive efficiency, Min *et al*. (2003b) observed that sheep grazing *Lotus corniculatus* increased their production of lambs by 25% due to increased rates of ovulation, and subsequently increased lambing percentage, possibly related to protein utilisation.

Luque *et al*. (2000) observed that ewes grazing on *Lotus corniculatus* had an ovulation rate up to 14% higher than ewes grazing pasture, with neither parameter being affected by PEG supplementation. Grazing of high CT-containing pastures, such as *Lotus corniculatus*, have been suggested to yield higher reproductive efficiency in sheep compared with animals grazed on grass alone (Ramirez-Restrepo and Barry, 2005).

4.6 Animal health

4.6.1 Bloat

It is many years since bloat, to which cattle, sheep, goats, and probably all other ruminants are subject, was first described (Cole *et al*., 1945; Jones *et al*., 1973). Bloat occurs when grazing animals consume large quantities of high nutritive value legumes such as lucerne (*Medicano sativa*) or white clover (*Trifolium repens*) (Aerts *et al*., 1999; Min *et al*., 2003b; Frutos *et al*., 2004). This disorder arises because the gases produced in the rumen during fermentation cannot be released in the normal way, since they are trapped in persistent foam due to the rapid release of soluble proteins during chewing and ruminal degradation (Frutos *et al.*, 2004; Rochfort *et al*., 2009). Bloat can proceed very rapidly and lead to crushing of vital organs and the ultimate death of the animal. The bloat safety of a number of legume forages is generally attributed to the presence of CT (Lees, 1992; Zimmer and Cordesse, 1996). The aggregation of soluble proteins with CT interferes with the formation of stable foam and is often sufficient to prevent bloat or reduce its severity. It is confirmed that the content of dietary CT needed to achieve bloat protection is 5 gCT/kg DM, but this concentration will depend on the nutrition, rate of eating and dietary composition (Waghorn and McNabb, 2003).

4.6.2 Gastro-intestinal parasites

Control of ruminant gastrointestinal parasites (GIP) over the past decades has been achieved by the use of anthelmintic drugs, but control of GIP is becoming more difficult due to the increased resistance of parasites to common anthelmintics, which has been reported in goats, sheep, and cattle (Pomroy *et al*., 2002), and the public concern about both the use of chemical treatments in farm production and the risk of residues in food products. Alternative parasite management strategies using forages containing condensed tannins (CT) have been advocated in many studies (Githiori *et al*., 2006).

The effects vary with the species of plant, and the concentration and structure of the CT present in the different plant species, the parasite and the host seem to be major factors modulating efficacy against nematodes (Hoste *et al*., 2006).

Two main hypotheses have been proposed to explain the effect of tannins against gastrointestinal nematodes in ruminants. Firstly, tannins could act indirectly, by improving the response of the host to parasites. The improved utilisation of nutrients by hosts receiving moderate amounts of CT could help improve the resilience usually observed in infected animals, and it could also modulate host resistance. Second, the direct hypothesis is that tannins could have their own anthelmintic properties that affect several key biological processes (Waghorn and McNabb, 2003; Hoste *et al*., 2006). However, the exact mechanisms of action remain obscure and could differ depending on the parasite, its stage of development and possibly the biochemical characteristics of the forage species (Min and Hart, 2003a).

Niezen *et al*., 2002 investigated the efficacy of the legume *Hedysarum coronarium* (sulla) for reducing gastrointestinal nematode infections, relative to lucerne. Diet did not affect faecal egg counts, but feeding sulla decreased numbers of *T. colubriformis* in uninfected lambs.

A study of sheep grazing lucerne or *Lotus pedunculatus* did not show any significant differences when PEG was added to the diet. By contrast, adding PEG to the diet of browsing goats led to significant increases in egg excretion and negative effects on growth, suggesting that tannins were involved in the antiparasitic effect (Kabasa *et al*., 2000).

The anthelmintic activity of some Mediterranean browse plants (*Pistacia lentiscus*, *Quercus coccifera*, *Onobrychis viciifolia* or *Medicago sativa*) against parasitic nematodes were studied by Manolaraki *et al*., 2010 on lambs. They found that consumption of the four browser plants did not cause any differences in pathophysiological measurements, but there were significant decreases in egg excretion, mainly explained by significant decreases in worm fecundity for all species, without any statistical difference in worm numbers. Also, Heckendorn *et al*., 2007 observed that compared with a ryegrass-lucerne mixture (control), administration of tanniniferous forages (chicory, birdsfoot trefoil, sainfoin) was associated with significant reductions of total daily faecal egg output specific to *H. contortus* and a tendentially reduced *H. contortus* worm burden.

In addition, some tropical legumes have shown promising results. Grazing of *L. cuneata* forage (50 gCT/kg DM achieved remarkably high reductions (57-100%) in faecal egg counts, total faecal egg output and the numbers of parasitic nematodes (*Haemonchus* and *Trichostrongylus*) in goats (Min and Hart, 2003a). High levels of dried *Acacia karoo* leaves (240 gCT/kg DM) significantly reduced faecal egg counts and *Haemonchus contortus* worm burdens in goats (Kahiya *et al*., 2003).

4.7 Environmental impact of condensed tannins

Ruminants are important producers of greenhouse gases such as nitrous oxide (N_2O) and methane (CH_4). The production of greenhouse gases (GHG) from livestock and their impact on climate changes are a major concern worldwide (Steinfeld *et al*., 2006).

4.7.1 Nitrogen emissions

Many studies have demonstrated that condensed tannins result in a pronounced shift in N excretion from urine to faeces (Misselbrook *et al*., 2005; Mueller-Harvey, 2006), which demonstrates that tannins have a direct impact on environmental N emissions. Faecal N is thought to be an environmentally less harmful form of N, as it is more likely to contribute to the accumulation of soil organic matter than urinary N (Whitmore and Handayanto, 1997; Grabber *et al*., 2001). However, urinary urea is rapidly converted into ammonia and then to volatile nitrous oxides, which are potent (GHG) (Givens and Rulquin, 2004; Kebreab *et al*., 2004). Therefore, lower urinary urea contents mean less volatile N losses into the environment. Furthermore, Misselbrook *et al*., 2005 compared tannin-free alfalfa and tannin-containing *Lotus corniculatus* diets and demonstrated that *Lotus corniculatus* tannins appeared to remain active in the faeces from cattle: they recorded reduced ammonia emissions from slurries on the barn floor and from slurries applied to the soil.

4.7.2 Methane emissions

Methane (CH_4) is one of the three main GHG together with carbon dioxide (CO_2) and nitrous oxide (N_2O). It is reported that CH_4 is the most abundant GHG emitted (50-60%) at the farm scale in ruminant production systems. This gas produced as a by-product of the digestive process and represents a significant energy loss from the animal, ranging from 2–12% of gross energy intake (Rochfort *et al*., 2009; Martin *et al*., 2010).

There have recently been reports of decreased CH_4 emission by ruminants consuming CT-containing forage (Puchala *et al*., 2005) and also when a supplemental CT extract was added to a forage–based diet (Carulla *et al.,* 2005).

Two modes of action of CT on methanogenesis have been proposed: (i) a direct effect on ruminal methanogenic bacteria, archaea, and (ii) an indirect effect on hydrogen production, which is a substrate for these bacteria, due to lower feed degradation, (Tavendale *et al*., 2005). Carulla *et al*. (2005) suggest that inhibition of methanogenesis by CT is primary the result of suppressed fibre degradation, limiting H_2 derived from the synthesis of acetate. Depressed fibre degradation could be due to a reduced number of cellulolytic bacteria, formation of tannin-cellulose complexes and (or) impaired bacterial adhesion to substrate and fibrolytic activity of rumen microbes. The same authors state that supplementation of *Acacia mearnsii* tannins at a level of approximately 0.025% of the diet DM significantly reduced methane emissions by 13%.

Animut *et al*. (2007) used wethers to assess effects on CH_4 emission of CT from different sources such as *Lespedeza striata*, *Lespedeza cuneata* and a mixture of them. They found that CH_4 emissions in l/day were markedly higher with PEG supplementation (19.1 l/day) *vs.* diet given alone (9.0 l/day). Also, Tiemann *et al*. (2008) reported that the inclusion of the tannin-rich shrub legume species *Callinadra calothyrsus* and *Fleminga macrophylla* in the diet reduced CH_4 emissions in growing lambs by up to 24%, but this was associated with reduced organic matter and fibre digestibility. De Oliveira *et al*. (2009) observed a lower ruminal apparent digested matter of NDF in high tannin diets (1.0 gCT/kg DM) and no effect of tannin levels on digestibility and methane emission.

It is believed that appropriately dosed tannins would reduce methane production, but not digestibility (Mueller-Harvey, 2006).

Woodward *et al*. (1994) conducted a trial to measure methane production by dairy cows grazing either good quality ryegrass pasture or *Lotus corniculatus*. The CT in lotus reduced methane production by 13% relative to lotus when the CT had been inactivated by PEG, and was responsible for 66% of the difference between lotus and ryegrass.

II. Sainfoin (*Onobrychis viciifolia*)

1. Agronomical value

1.1 Identification

Sainfoin is a perennial, forage and fodder legume plant, originating from the eastern Mediterranean. It belongs to the genus *Onobrychis* and there are some 130 different Eurasian species of *Onobrychis*, but the main cultivated species is *Onobrychis viciifolia* Scop. As a legume, it is able to convert inorganic gaseous atmospheric nitrogen into bioavailable nitrogen compounds. *Onobrychis viciifolia* can also be used by grazing animals as harvested for feeding animals.

The English term sainfoin is derived from the French *sain foin* which means wholesome hay and the name of the genus, *Onobrychis*, is a Greek term meaning 'donkeys' favourite fodder'.

Sainfoin has been documented for at least 2000 years, has been grown in parts of Europe and Asia and is used in the western United States and Canada.

The cultivation of sainfoin has declined steadily until today in Europe. In France, from 700 000 ha in 1940, crop surface declined to 400 000 ha in 1960 and 20 000 ha in 1986 ha almost all exclusively located in south-east France (Prosperi *et al*., 1994). Nowadays, in France, sainfoin represents 0.1% of the total surface for artificial grasslands corresponding to the cultivation of leguminous forages (http://agreste.agriculture.gouv.fr/).

It was widely grown in England during the 17th-19th centuries and to a lesser extent in the early 20th century, but it is rarely used in New Zealand (Burton and Curley, 1968; Waghorn *et al.*, 1998). This plant is still being cropped in Italy, Spain and Iran and over 94 000 ha of sainfoin is grown in Turkey (Eken *et al*., 2004).

A decline in sainfoin culture has been observed over the last 40 years throughout Europe. This happened basically because of its low persistence in the stand and low regrowth rate after the first spring cut (Borreani *et al*., 2003). In France, one of the reasons for this decline was the cessation of research into and selection of sainfoin (Prosperti *et al*., 1994). The reduction of 70% especially in the area grown in Italy was due to structural changes in Italian agriculture and the gradual disappearance of livestock farms in hilly areas (Borreani *et al*., 2003). Also, forage legume use in Europe has decreased substantially in recent years, as European agricultural policy supports farmers with payments, towards relatively intensive production, with reliance on inorganic nitrogen (N) fertilizers, rather than low-input systems (Rochon *et al*., 2004).

1.2 Morphology

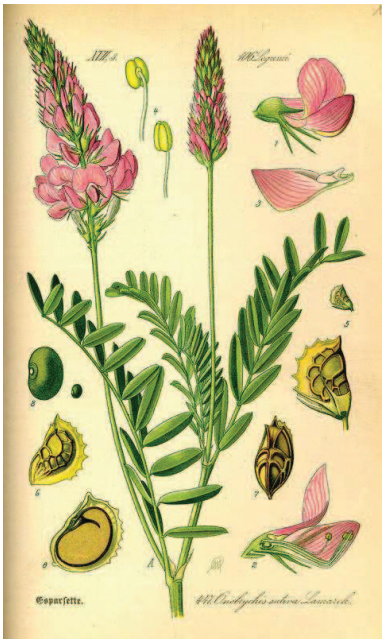
Sainfoin is a perennial plant with numerous erect stems arising from basal buds, forming a branched crown. Each stem has pinnate leaves formed of 10-28 leaflets grouped in pairs on

long petioles and with a terminal leaflet. Each flower can produce a kidney-shaped seed contained in a brown pod http://caliban.mpiz-koeln.mpg.de/thome/band3/tafel_131.html) (**Figure 11**). It grows from 40 to 100 cm in height. The root system consists of a stout tap-root with a few main branches and numerous fine lateral roots (Spedding and Diekmahns, 1972; Frame *et al*., 1998).

A range of types exists, but these are mainly local selections rather than bred cultivars. *Onobrychis viciifolia* has been divided into two agricultural types: 'common sainfoin' and 'giant sainfoin'. The common type is also named single-cut as it produces one conservation cut per year. The giant type has the ability to reflower after being cut, has longer stems, less stem per plant and more upright stems than those of common sainfoin (Spedding and Diekmahns, 1972).

1.3 Climate and soil requirements

O. vicifoilia is adapted to a wide range of climate conditions and grows well on a variety of soils. Sainfoin is best suited to neutral and alkaline soils of pH 6 or above and also to dry land and irrigated areas, similar to *Medicago sativa (*Spedding and Diekmahs, 1972; Waghorn *et al*., 1998*;* Cash *et al*., 2006). *Onobrychis viciifolia* is intolerant of water-logging and so the soil needs to be drained (Sheldrick *et al.,* 1987)*.* Forage legumes are most tolerant to freezing temperatures and the *O.viciifolia* seedlings seem to be more resistant than *Medicago sativa* and than most of the *Trifolium* species (Meyer and Badaruddin, 2001). The seed-bed should be free of rhizomatous weeds and soils should be tested and amended with appropriate levels of phosphorus and potassium prior to planting. Application of 40 kg P and 120 kg K/ha is recommended (Spedding and Diekmahns, 1972)





Variety: Perly

Figure 11: Sainfoin (*Onobrychis viciifolia*).

1.4 Use of sainfoin

1.4.1 **Pasture** (alone or in a grass-legume mix)

Sainfoin has been introduced in pasture, alone or in a grass-legume mix, and its advantage for pasture use includes excellent quality and palatability without the danger of bloat. Sainfoin-grass mixtures generally have higher yields and quality than grasses alone. A study of sainfoin sown alone or mixed with grasses in Turkey found that sainfoin grass mixtures produced higher yields than a sainfoin monoculture (Sengul, 2003). For mixtures, sainfoin and grass should be planted in alternate rows. Once established, sainfoin maintains itself well in mixed vegetation in dryland areas. When *O.viciifolia* was used for grazing, light grazing only in the late autumn was suggested, to allow the crop time to replenish root reserves (Sheldrick *et al.,* 1987).

1.4.2 Conserved forage: hay and silage

Sainfoin can be used mainly as a hay crop or for pasture, but it can be cut for silage as well (Bland, 1971; Sheldrick, 1987; Waghorn *et al.,* 1998). It is well suited to haying due to its upright growth habit and is easiness to cut. First cut hay yields are often greater than those for alfalfa, but later cuttings commonly yield less than alfalfa (Baldridge and Lohmiller, 1990).

When harvested as silage, the tannins present in sainfoin can reduce the proteolysis that takes place in the silo (Albrecht and Muck, 1991; Salawu *et al*., 1997; Wilkins and Jones, 2000).

The potential value of co-ensiling alfalfa with the CT-containing legume sainfoin (*Onobrychis viciifolia*) was investigated by assessing the silage nutritive quality as well as ruminal and total tract digestion of the ensiled mixture. Incorporation of sainfoin into alfalfa forage improved fermentation in laboratory-scale silos, and the alfalfa:sainfoin ratio yielding optimal ensiling and ruminal fermentation was approximately 60:40 (DM basis) (Wang *et al*., 2007).
Sainfoin silages showed good ensiling characteristics and had relatively high concentrations of undegraded protein, when protein metabolism was studied during ensiling of three different sainfoin varieties (Lorenz *et al*., 2010).

1.5 Dry matter (DM) yield

Depending on growing conditions, dry matter yield of *O.viciifolia* may vary. Research was carried out in 1998–2000 and observed that DM yield increased from 0.5 t ha^{-1} in the early vegetative stages to 8 t·ha^{-1} in the late flowering stage without any significant difference over the years (Borreani *et al*., 2003). The DM yield obtain during the first vegetation cycle is higher compare to the following cycles. Delgado *et al*. (2010) supported that two thirds of the forage yield are obtained from the first cut in spring that is used for haymaking.
the remaining production is distributed between one or two light regrowths in the summer-autumn period. Yields are about 20% lower than those of *Medicago sativa*. The contributing factors are mainly lower leaf area indices (LAI), less canopy structure and less efficient gaseous nitrogen fixation (Frame *et al*., 1998). Liu *et al*. (2008a) reported that in a field experiment run for 3 years in the UK, the annual yield of a monoculture of sainfoin (*O.viciifolia*) was 4.53 t DM ha^{-1} and that of sainfoin-grass mixtures was 8.33 t DM ha^{-1} averaged over 3 years. Both sainfoin and sainfoin-grass mixtures had higher annual DM yields than the grass monocultures. Sowings of *O.viciifolia* from April to July had similar yields of about 9.0 t DM ha^{-1}. According to Liu *et al*., 2008b the most reliable month for sowing is April. August and September proved to be unsuitable sowing times, producing less herbage in subsequent years compared with April-July sowing.

1.6 Diseases and insects

It is reported that sainfoin (*O.viciifolia*) crop is relatively free from serious disease and insect problems compared with other legumes, and it is resistant to many of the pests that attack other forage legumes (Morrill *et al*., 1998).

2. Nutritive value

Despite several studies on nutritive value and CT contents and structures of *L.* species (Foo *et al*., 1982; Hedqvist *et al*., 2000; Min *et al*., 2003b; Meagher *et al*., 2004) there are only a few reports characterising sainfoin CT and its nutritive value.

2.1 Chemical composition

Immature sainfoin contains 34 N g/kg DM (21 %CP), and soluble carbohydrate content can exceed 20% of DM (Waghorn *et al*., 1998). A notable feature of sainfoin is the content of condensed tannins in its leaves. Digestibility of DM is about 70% (Waghorn *et al*., 1998). Sainfoin is mineral-rich in comparison with grasses, but its Ca and Na concentrations are lower than that of other major legumes (Spedding and Diekmahns, 1972). The protein score, an estimation of protein quality for non-ruminants, was 68 for sainfoin forage and 71 for lucerne forage compared with 100 for an 'ideal protein'. The similarity of protein quality of sainfoin forage to that of lucerne indicates that sainfoin could be grown as an alternative to other crops for the production of protein (Kaldy *et al*., 1979).

2.2 Content and structure of sainfoin CT

Not a lot of research has been carried out on the condensed tannins found in sainfoin. The CT comprise about 5-8% of DM (depending always on the analysis method) and may be less detrimental to digestion than those from *Lotus* (Waghorn *et al*., 1998). In comparison, *Lotus corniculatus* and *Lotus pedunculatus* differ in their CT contents (Terrill *et al*., 1992) and in their chemical structures (Meagher *et al*., 2004). The PD:PC ratio was 16:84 for *Lotus corniculatus* and 70:30 for *Lotus pedunculatus* (Foo *et al*., 1982; Hedqvist *et al*., 2000). Therefore, sainfoin CT structures appear to resemble *Lotus pedunculatus* CT (Foo *et al*., 1982; Marais *et al*., 2000), but the nutritive value of sainfoin is reportedly much higher than *Lotus pedunculatus* as reviewed by Min *et al*. (2003b). The content and composition of sainfoin CT change with leaf age (Koupai-Abyazani *et al*., 1993b), and their ability to bind protein tends to be higher in less mature forage plants (Bate-Smith, 1973). According to Marais *et al*. (2000), PD:PC ratios in different sainfoin varieties range from 36:64 to 93:7. Lees *et al*. (1995) found that sainfoin cells lost tannins during maturation, and the authors concluded that condensed tannins were catabolised during plant maturation and senescence.

2.3 Palatability and voluntary feed intake

It has been found that condensed tannins reduce the palatability and intake of feeds (Frutos *et al*., 2004). Both grazed and conserved sainfoin and sainfoin-rich mixed forages are highly acceptable, leading to high animal intakes of forage (Griggs and Matches, 1991). Voluntary intake of *O.viciifolia* was found to be 20-24% higher than intake of grasses, and 10-29% higher than intake of *Trifolium pratense* or *Medicago sativa* (Waghorn *et al*., 1990). However, voluntary intake of sainfoin was higher than that of lucerne until the start of flowering and declined beyond that phenological stage (Tables INRA, 2007). In a palatability study, 6-month old heifers preferred sainfoin hay to alfalfa hay (Parker and Moss, 1980). Aufrère *et al*., 2008 measured voluntary intake in sheep fed sainfoin and lucerne as fresh or hay and found that it was similar on fresh forages and higher for lucerne hay. However, Fraser *et al*., 2000 found that voluntary intakes were similar on the *Lotus*, sainfoin and late-cut red clover silages, but the voluntary intake on the *Lotus* silage was significantly higher than that on the lucerne silages and early-cut red clover silage. Scharenberg *et al*., 2007 found that sainfoin, although it had a high CT content (up to 100 g/kg DM), was more palatable than the more popular *Lotus corniculatus* with a lower CT content (Mueller-Harvey, 2006). Although sainfoin stems appear to be coarse, they remain succulent well into maturity and are more digestible than alfalfa stems. The crop is palatable to all classes of livestock as either hay or pasture.

2.4 Rumen degradation

One of the main characteristics of forage legumes contaning CT is that they bind dietary proteins in the rumen and thus reduce excessive protein degradation by micro-organisms (Waghorn *et al*., 1998; Aerts *et a*l., 1999; Turner and Neel, 2003; Mueller-Harvey, 2006). Aufrere *et al*., 2008 reported that the rumen degradation of N was lower in sainfoin than in lucerne studied as either fresh or hay, resulting in a lower proportion of N intake excreted in urine. Clarke and Reid, 1974 reported that when fresh sainfoin was fed to sheep, the site of nitrogen digestion was transferred from the rumen to the intestines, and the N digestibility was as high as for lucerne.

Sainfoin condensed tannins were found to decrease soluble N and NH_3-N content in the rumen fluid, as shown in sheep (Waghorn *et al*., 1990) and in an *in vitro* continuous culture system (Dahlberg *et al*., 1988). Barry and McNabb (1999) reported that fresh *O.viciifolia* decreased ruminal ammonia concentration, and Scharenberg *et al.*, 2007 reported the same on feeding dehydrated and ensiled *O.viciifolia* to lambs. Wang *et al*. (2006) found that cattle grazing *Medicago sativa-O.viciifolia* mixtures had lower ruminal ammonia concentrations than those grazing pure *Medicago sativa* pasture. With pure silage of *O.viciifolia* a decrease in ammonia concentration was observed (Wang *et al*., 2007). CT of sainfoin fed as fresh forage to sheep (Theodoridou *et al*., 2010) or ensiled to lambs (Scharenberg *et al*, 2007) modified the partition of N excretion by reduced urine N losses and increased N in faeces without impacting the N

retention in the animals. Egan and Ulyatt (1980) reported that retained N (g/d) was halved with a late-cut sainfoin compared with an early-cut sainfoin.

2.5 Digestion in small intestine

Sheep fed dried sainfoin had a greater flow of amino acids to the duodenum than sheep fed dried lucerne (Harrison *et al*., 1973).

In sheep fed four fresh herbage diets, ryegrass, white clover and sainfoin, no significant differences were observed between herbage species or intake in the percentage of digested N in the stomach or intestines. The CT in sainfoin had no effect on nitrogen digestion (Ulyatt and Egan, 1979).

Although sainfoin leaf CT had a similar structure to *Lotus pedunculatus* (in terms of PD:PC ratio (70:30)), their effect on intestinal absorption clearly differed. Bermingham *et al*., 2001 found that CT in sainfoin (*Onobrychis viciifolia*) had no significant effect on apparent absorption of essential amino acids from the small intestine.

2.6 *In vivo* digestibility

Bermingham *et al*., 2001 and Scharenberg *et al*., 2007 on sainfoin observed that CT decreased organic matter digestibility (OMD). However, OMD was not modified by the presence of sainfoin CT compared with lucerne (Aufrère *et al*., 2008) or between lucerne, *Lotus corniculatus* and sainfoin compared as hays or silage (Kraim *et al*., 1990).

2.7 Nitrogen retention and retention

An increase in faecal nitrogen excretion and a decrease in urinary nitrogen, a typical effect of CT, (Hervas *et al*., 2004; Waghorn, 2008) was observed in cows fed sainfoin (Scharenberg *et al*, 2009). Lambs fed dehydrated or ensiled sainfoin had a higher faecal excretion, compensated by lower urine-N amounts, whereas no significant differences were observed in body N retention (Scharenberg *et al*., 2007).

2.8 Animal performance

Sainfoin gives highly satisfactory individual animal performance. Among other legumes, only white clover was superior to sainfoin as judged by lamb growth (Ulyatt, 1981).

2.9 Animal health

2.9.1 Bloat

Sainfoin is a non-bloating perennial forage and does not cause bloat in animals (Waghorn *et al.*, 1990; Barry and McNabb, 1999; Mc Machon *et al*., 2000). Animal deaths from bloat were also reduced on a mixed pasture of alfalfa/sainfoin (9:1), which amounted to 2–5 gCT/kg DM in

the diet (McMahon *et al*., 2000. A grass/alfalfa pasture with 10% dock (*R. obtusifolius*), or 2 gCT/kg DM in the diet, also prevented bloat (Waghorn, 1990). The bloat-safe feature of *O.viciifolia* was confirmed in a trial where it was shown that every *Medicago sativa* cultural crop tested caused bloat, but sainfoin (*Onobrychis viciifolia*), birdsfoot trefoil (*Lotus corniculatus*), and cicer milkvetch (*Astragalus cicer*) did not induce bloat (Majak *et al*., 1995).

2.9.2 Anthelmintic properties

Condensed tannin-containing forages, such as sainfoin, have considerable potential to act as direct antiparasitic agents, but may also act indirectly by increasing host resistance (Hoste *et al*., 2006)

Results of *in vitro* assays comparing the activity of specific biochemical fractions obtained from sainfoin confirmed that the antiparasitic activity, measured by the inhibition of larval migration (LMIA), was associated mainly with the fractions containing condensed tannins (Barrau *et al*., 2005). *O.viciifolia* has been shown to have an antiparasitic effect on the most important sheep nematode, *Haemonchus contortus* (Heckendorn *et al*., 2006). *O.viciifolia* has been also shown to have a negative effect on nematode egg excretion and a positive effect on host resilience in goats (Hoste *et al*., 2005; Paolini *et al*., 2005).

However, Scharenber *et al*., 2008 found that when lambs fed sainfoin, either untreated or treated with PEG, CT exerted no beneficial effects on resilience to nematode infection. Also, faecal egg count, worm burden, and packed cell volume were not affected by the greater crude protein supply associated with sainfoin feeding. They concluded that the lack of effects of sainfoin on resilience to nematode infection might have been the result of the unexpectedly low CT content (3.6% in DM) of the forage.

Brunet et al., 2008, tested the effect of sainfoin extract on the association of *H. contortus* larvae with abomasal tissue. From this *in vitro* study, they found that following 3 h contact with sainfoin extract at 1,200 µg/ml, the penetration of exsheathed L3s of *H. contortus* and *Teladorsagia circumcincta* into fundic explants was significantly reduced. Also, a study was performed *in vivo* on rumen-cannulated sheep fed with different proportions of sainfoin in the diet. The consumption of a higher proportion of sainfoin was indeed associated with significant delays in *Haemonchus* exsheathment. The authors concluded that interference with the early step of nematode infection might be one of the modes of action contributing to the anthelmintic properties of tanniniferous plants (Brunet *et al*., 2007).

Table 3: Effect of condensed tannins on rumen function (Waghorn, *et al*., 1999)

Low dietary contents of CT with temperate forages (to 50 g/kg DM)
• Reduced (20-40 %) ammonia concentration
• Reduced proteolysis of plant protein and apparent N digestion in the rumen
• Possible minor reductions in rate of fermentation
• Minimal effect of pool size, turnover effect or voluntary intake
• No effect on digestibility of structural fibre
• Minimal effect on microbial outflow to the small intestine (SI)
• Increased availability of amino acids (AA) from the SI
Medium dietary contents of CT with temperate forages (50-120 g/kg DM)
• Substantial reduction (30-70%) in ammonia concentration
• Reduced proteolysis of plant protein and apparent N digestion in the rumen
• Reduced rate of fermentation, slower turnover rate and large rumen pool size, 20-40% reduction in VFA concentration
• Reduced voluntary food intake (10-30%)
• Minor reductions in digestibility of structural fibre
• Reduced microbial flow to SI, reduced total AA flow to SI because of intake depression

Table 4: Effect of condensed tannins on intestinal function in ruminants (Waghorn, *et al*., 1999)

Low-medium dietary contents of CT (to 80 g/kg DM)
• Mucous production increased to protect intestine
• Excess mucous slows movement of metabolites to enterocytes for absorption
• Some CT will remain associated with digesta constituents through intestine and inhibit enzymic hydrolysis
• Possible increase in the enzyme excretion
• Small increase in endogenous protein losses
• Small (up to 20%) reductions in apparent digestibility of AA
Medium-high dietary contents of CT (over 80 g/kg DM)
• High rate of mucous production which may be insufficient to protect the intestine
• Physical damage to the brush border region of intestine, lesions and leakage of endogenous protein
• Some CT will remain bound to digesta constituents and remainder will bind to endogenous mucous, enzyme and intestinal tissues
• Depressed absorption of nutrients
• High rate of endogenous protein loss
• Significant reduction in AA digestibility
• Death after prolonged feeding

Concluding remarks

Consumption of condensed tannins by ruminants has been associated with both positive and negative effects. Positive effects of CT regarding the different zootechnic parameters are higher wool growth, live weight gain, milk yield and quality, meat quality, reproductive efficiency and prevention of bloat and parasites. From the nutritive point of view, positive effects include protein protection from excessive fermentation in the rumen, potentially increasing protein absorption and increased body nitrogen retention. Negative effects can include reduced voluntary feed intake and palatability, and reduced fibre and dry matter digestibility due to impaired rumen function. The effects of CT depend on factors such as the structure of tannins and their content in plants. For that reason, nutritive and anthelmintic benefits and the potential of CT for animal performance can be achieved only when the composition, structure and biological function of CT have been well defined.

Chapter 3

Experimental work

PART 1

In vivo and *in situ* effect of condensed tannins in fresh sainfoin on digestive and nitrogen balance and on the ruminal and intestinal digestion parameters

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Effects of condensed tannins in fresh sainfoin (*Onobrychis viciifolia*) on *in vivo* and *in situ* digestion in sheep

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Abstract

This study focused on effects of structure, content and biological activity of condensed tannins (CT) in leaves, stems and whole plant of sainfoin (*Onobrychis viciifolia)* on its *in vivo* and *in situ* digestive characteristics in sheep. Sainfoin was studied as fresh forage during the first vegetation cycle at two phenological stages (*i.e.*, end of flowering and green seeds) and during the second vegetation cycle (*i.e.*, start of flowering). The feeding experiment used 12 sheep; with six dosed, through the rumen cannula, with polyethylene glycol (PEG) to neutralise CT effects. Organic matter digestibility (OMD), total tract N disappearance and N balance were measured in sheep fed the whole plant. The residues of dry matter (DM) and N from nylon bags suspended in the rumen were determined on leaves and stems. Intestinal digestibility was measured using other, intestinally fistulated sheep. PEG addition and vegetation cycle increased total tract N digestibility ($P<0.001$) but PEG affected OMD only at the end of flowering. PEG inactivated the CT and increased urinary N excretion ($P<0.05$) but this was offset by lower faecal N excretion ($P<0.001$). Feeding sainfoin can be used to alter the form of excreted N (*i.e.,* urine versus faeces) and thus potentially reduce environmental N pollution without affecting body N retention. Kinetic studies of total N, ammonia N (NH_3-N) and volatile fatty acids (VFA) in rumen fluid were made before and 1.5, 3 and 6 h after feeding. Sainfoin CT decreased rumen fluid soluble N ($P<0.05$) and NH_3-N ($P<0.01$). Ruminal N disappearance (DisN) of leaves or stems was lower in the presence of active CT compared to PEG-inactivated CT ($P<0.001$) for both vegetation cycles. PEG also increased intestinal digestibility ($P<0.05$) of leaves and stems. Leaves had lower ruminal DisN, but higher N disappearing from intestine than stems. The biological activity and content of CT in the whole plant decreased as phenological stage increased. Prodelphinidin:procyanidin (PD:PC) ratios of leaves varied with vegetation cycle and phenological stage. The molecular size of CT in the whole plant, as indicated by their mean degree of polymerisation (mDP), was lowest at the start of flowering and coincided with the higher biological activity and content of CT.

Abbreviations: '*a*', rapidly disappearing fraction; ADF, acid detergent fibre expressed inclusive of residual ash; '*b*', slowly disappearing fraction; '*c*', disappearance rate; CT, condensed tannins; DisDM, DM disappearance; DisN, N disappearance; DM, dry matter; DOM, digestible OM; ha, hectare; mDP, mean degree of polymerisation; aNDF, neutral detergent fibre assayed with a heat stable amylase and expressed inclusive of residual ash; NH_3-N, ammonia N; OMD, OM digestibility; PEG, polyethylene glycol; PC, procyanidins; PD, prodelphinidins; Phs, phenological stage; RDA, radial diffusion assay; sa, sulphuric acid; SI, small intestine, t, tonne; T, treatment; VFA, volatile fatty acids; BW, body weight; $BW^{0.75}$, metabolic BW.

Keywords: Sainfoin Feed Value, Sheep, *in vivo* and *in situ* measurements, Tannins, Polyethylene Glycol

1. Introduction

The current worldwide emphasis on environmental issues requires that animal feeding systems use less chemical inputs to ensure more sustainable farming systems. Forage legumes can increase the N content of ruminant diets and require less fertiliser to grow. However, proteins in forage legumes are often poorly utilised by ruminants because of their extensive, rapid degradation in the rumen, which leads to N losses via urinary urea excretion (Min et al., 2003). Better N utilisation is needed for more efficient and environmentally sustainable ruminant feeding systems. Utilisation of forage proteins can be optimised if a higher proportion of protein survives ruminal degradation and passes into the lower digestive tract, where it can be absorbed. One method for improving N utilisation by ruminants is to use forage plants that contain CT, which are polyphenols which bind proteins to protect them from degradation in the rumen (Waghorn et al., 1987; Mueller-Harvey and McAllan, 1992; McNabb et al., 1996). Despite numerous studies describing effects of CT on the nutritive value of plants (Barahona et al., 1997; Barry and McNabb, 1999; Waghorn, 2008), few have

assessed these CT structures (Schofield et al., 2001; Meagher et al., 2004; Mueller-Harvey, 2006) relative to how they might differ between phenological stages and plant parts.

The aim was to characterise the CT in the leaves, stems and whole plant of sainfoin (*Onobrychis viciifolia*) at three phenological stages and two vegetation cycles in order to investigate effects of CT on *in vivo* digestibility, N balance and ruminal digestive parameters in sheep, as well as on ruminal and intestinal disappearance *in situ*. To assess effects of sainfoin CT, measurements were made with and without polyethylene glycol (PEG), which inactivates CT throughout the digestive tract (Waghorn and Shelton, 1997; Barry et al., 2001; Makkar, 2003).

2. Materials and methods

2.1. Forages

Sainfoin (*Onobrychis viciifolia*) cv. Perly was grown in Clermont-Ferrand (France), at INRA's Crouël site at an altitude of 320 m, on a fertile, basic loamy soil (374 g/kg sand, 458 g/kg loam, 168 g/kg clay), 37g/kg organic matter, pH 8.2. No mineral fertiliser was applied. Experiments were conducted during 2008 with fresh forages harvested at two phenological stages from the first vegetation cycle and at one stage from the second vegetation cycle. Stage 1 was studied from 2 to 11 June and stage 2 from 16 to 25 June. Stage 3 was studied from 7 to 16 July, (*i.e.*, five weeks after stage 1). Stage 1 represented the end of flowering (*i.e.*, 1/2 of stems showed open flowers on the upper half of the flower stem), stage 2 contained green seeds (*i.e.*, 1/2 of floral stems showed green seeds without open flowers), and stage 3 represented the start of flowering (*i.e.*, 1/2 of stems showed open flowers on the lower half of the flower stem). The yield of sainfoin was 8.5 t DM/ha $\pm$ 1.1 at stage 1, 8.5 t DM/ha $\pm$ 0.8 at stage 2 and 3.3 t DM/ha $\pm$ 0.4 at stage 3.

2.2. Animals and experimental design

The study was conducted indoors at the INRA-UR1213 experimental farm in France (45°42'N, 03°30'E). The sheep were handled by specialist personnel trained in animal care and welfare according to the European Union Directive No. 609/1986 under agreement No. A63 565.

Two groups of six male castrated Texel sheep (12 months old, 60 ± 3.2 kg body weight(BW)) fitted with rumen cannulae were used for digestibility and N balance measurements (Demarquilly et al., 1995), sampling of ruminal fluid, and for measurement of ruminal disappearance using a nylon bag technique (Michalet-Doreau et al., 1987 as modified by Dulphy et al., 1999). Sheep were weighed before each experimental period and drenched with anthelmintic (Ivomec, Merial, Lyon, France) to eliminate internal and external parasites 30 days before the experiment began.

Sheep were offered 60 g DM of fresh sainfoin per kg metabolic $BW^{0.75}$ in two equal meals per day at 08:00 h and 16:00 h. One group was dosed daily with 60 g of PEG 4000 (2× 30 g in 200 ml water) delivered through the ruminal cannula before meals, and the other group was dosed with 2 x 200 ml of water without PEG.

Each of the three experimental periods (*i.e.*, one per phenological stage) comprised two weeks for adaptation followed by two weeks for measurements: one week to measure digestibility and N balance and sample ruminal fluid, and one week for *in situ* disappearance measurements (Fig. 1). During the two weeks of measurements, the sheep were housed in individual metabolic crates in which they had *ad libitum* access to water and salt block.

After the main experiment, which yielded residues of ruminal *in situ* degradability, another group of six adult Texel sheep, fitted with ruminal, duodenal and ileal T-type cannulae was used to measure intestinal digestibility by a mobile nylon bag technique. The six sheep were fed with fresh sainfoin with three receiving 60 g of PEG in 400 ml of water daily and the other three receiving 400 ml of water daily.

2.3. In vivo digestibility and N balance

The metabolic crates used for measurements of digestibility and N balance were equipped for individual daily collection of total faeces and urine. Loss of ammonia from urine was prevented by daily addition of 50 ml of 30 g/100 ml of sulphuric acid to the collection flask. Daily aliquots of faeces and urine were pooled per sheep during the weeks of measurement. Before feeding, fresh forages were weighed and fed to the sheep. Every day, feed samples and refusals were dried in a forced air oven at 60° C for 72 h for dry matter (DM) determination.

All samples were ground through a 1 mm sieve before analysis. Pooled samples of urine were stored at -20° C for later analysis of N.

2.4. Ruminal fluid sampling

Rumen fluid was sampled during four consecutive days from three sheep each day in order to sample all 12 sheep (*i.e.*, six with and six without PEG). Sampling was before the morning meal (*i.e.*, 0 h), and then 1.5, 3 and 6 h after feeding. At each time, 250 mL of rumen fluid was sampled, analysed for pH and muslin-filtered. After filtration, 3 ml of rumen fluid was mixed with 3 ml of a MFS (methylgreen – formalin saline) solution for determination of ruminal protozoa at 0 and at 3 h post feeding.

Rumen fluid was first centrifuged for 5 min at 3000 $\times$ *g* at 4°C to remove large feed particles. Samples for volatile fatty acids (VFA) analysis were obtained by taking 0.8 ml from the supernatant mixed with 0.5 ml of crotonic acid and then centrifuged at 14,000 $\times$ *g* for 10 min at 4°C. The supernatant of the first centrifugation of the rumen fluid was centrifuged again at 4°C and 27,000 $\times$ *g* for 20 min to remove small feed particles and bacteria. Total N and NH_3-N contents were measured in the supernatant.

2.5. In situ disappearance in the rumen

Ruminal degradation of DM and N was measured using a nylon bag procedure in which dacron bags (pore size 53 $\pm$ 15 μm, Ankom Co., Macedon, NY, USA) were used, with an internal surface

area of 5 × 11 cm and closed by heat sealing. For each phenological stage, after harvesting the sainfoin, stems and leaves were separated manually, cut into 1 cm lengths to mimic mastication of the sheep, and placed in the dacron bags. The bags were filled with leaves or stems with a quantity equivalent to 2.5 g DM and closed by heat-sealing. Bags of fresh leaves or stems of the forages were quickly frozen in liquid N and stored at -20°C before use.

In situ degradation was measured in the rumen of 12 sheep (*i.e.*, six with and six without PEG) with incubation times of 2, 4, 8, 16, 24 and 48 h. For one week, the dacron bags with leaves, and then with stems, were incubated in the rumen of the sheep. Each sheep first received two bags filled with leaves for the incubation times 2, 4 and 8 h, six bags filled with leaves for the incubation time 16 h, and three bags filled with leaves for the incubation times 24 and 48 h. The same procedure was followed for incubation of the dacron bags filled with stems. Samples of a common grass hay used as a 'standard' were incubated for 8 h in duplicate in the rumen of each sheep to detect any changes in the level of rumen degradation activity during the experiment. After removal from the rumen, bags were washed and stored at -20°C until analysis. Before analysis, the bags were thawed and washed in a washing machine (3 × 10 min) with cold water until the rinse water was clear. The bags were then beaten for 7 min in a "stomacher" to reduce microbial contamination of the undegraded fraction of the sample, washed again (2 × 10 min) and freeze-dried. A correction to account for adherent bacteria not detached by this treatment was performed according to Michalet-Doreau and Ould-Bah (1989).

2.6. In situ intestinal disappearance

In situ intestinal N disappearance of sainfoin was measured using a mobile dacron bag technique on six sheep fitted with duodenal and ileal cannulae. Leaf and stem residues recovered after 16 h of rumen incubation were considered to be representative of the forage residues escaping from the rumen and were used to fill the intestinal dacron bags (18 × 18 mm, 48 μm pore size, 100 to 120 mg per bag). Before filling the bags, residues recovered after 16 h of rumen incubation were

freeze-dried and ground to pass a 1 mm sieve. Bags were closed by heat sealing. Before placing them in the duodenum, bags were incubated for 1.5 h at 39°C in an HCl solution (0.01 M, pH 2.7) of pepsin (2g/L, 2000 FIP-U/g, Merck KGaA Darmstadt, Germany), to simulate abomasal digestion. After the morning meal, no more than eight bags per sheep were introduced into the duodenum, at a rate of one bag every 30 min. Three hours after placing the dacron bags in the duodenum, the ileal cannula was opened for bag recovery every 30 min. Recovered bags were briefly rinsed under cold water and stored at -20°C. After thawing, all bags were washed (5 $\underline{\times}$ 2 min) using a standardised procedure (*i.e.*, shaking in 1 L flasks containing 500 ml of warm water (39°C), and freeze-dried).

2.7. Laboratory analyses

N in the forages (*i.e.,* whole plant, leaves, stems), faeces, urine and rumen fluid were determined by a Kjeldahl method according to AOAC (1990, method 977.02). NH_3-N in rumen fluid was determined by the method of Weatherburn (1967). Residues from *in situ* incubations (*i.e.,* leaves, stems) were analysed for N by a Dumas method (Buckee 1994). Neutral detergent fibre (aNDF), acid detergent fibre (ADF) and lignin(sa) of the fresh forages, refusals, faeces, leaves and stems were analysed in filter bags according to Van Soest and Wine (1967) as modified by Pagan et al. (2009). Pepsin-cellulase digestibility of the forages (whole plant) was determined according to Aufrère and Michalet-Doreau (1983).

Tannins were analysed by direct thiolysis of freeze-dried samples with benzyl mercaptan at 40°C for 1 h to obtain quantitative and qualitative information on tannins (Guyot et al., 2001; Gu et al., 2003). Quantitative data refer to the content of extractable plus unextractable CT; where qualitative data provide information on tannin structures, such as, mean degree of polymerisation (mDP) (*i.e.,* molecular size), prodelphinidin:procyanidin (PD:PC) (*i.e.,* PD value) and *cis*:*trans (i.e., cis* value) ratios. The biological activity of CT in terms of protein-binding capacity was determined with the radial diffusion assay (RDA; Makkar et al., 2000). This assay measures precipitation of bovine serum albumin (BSA) by tannins, with the area of the precipitation ring being proportional to

the amount of extracted CT. For the RDA assay, CT were extracted with acetone/water (7:3, v/v) from freeze-dried leaves, stems or whole plants. VFA in the rumen liquor were analysed by gas chromatography (Jouany, 1982). Ruminal protozoa were enumerated using the counting chambers of Neubauer (Ogimoto and Imai, 1981).

2.8. Calculations

In situ DM and N disappearance curves in the rumen of leaves and stems were fitted to the model of Ørskov and McDonald (1979) using a non-linear regression procedure (SAS, 2000):

$$P = a + b\,(1 - e^{-ct})$$

where: 'P' is potential disappearance, '*a'* is the rapidly disappearing fraction, '*b'* is the slowly disappearing fraction, and '*c'* is the disappearance rate of the slowly disappearing fraction and (100 – *a* – *b*) was considered to be the residual fraction. The effective disappearance of N, DisN, was calculated as:

$$DisN = a + bc / (c + k_p)$$

assuming a passage rate 'k_p' = 0.06/h. The same models were used to calculate the effective disappearance of DM (DisDM), a, b and c parameters for DM.

2.9. Statistical analysis

Data were analysed using the Mixed Procedure of the SAS (2000). Data related to forage chemical composition were analysed according to a model including phenological stage. Data from *in vivo* and *in situ* measurements were subjected to ANOVA to test phenological stage (Phs) and treatment (T) (with or without PEG) as fixed effects, with sheep within treatment as a random effect. Data of rumen fluid were subjected to ANOVA according to a repeated analysis measurement. Time was considered to be a repeated variable.

Orthogonal contrasts were used to compare difference between phenological stages (*i.e.,* end of flowering *versus* green seeds stages) and between vegetation cycles (*i.e.,* end of flowering *versus* start of flowering and green seeds *versus* start of flowering).

3. Results

3.1. Chemical composition of the forages

The N content of leaves, stems, and of the whole sainfoin plant was higher (P<0.001) at the start of flowering than at the more advanced maturity stages (Table 1). The fibre content (aNDF and ADF) of sainfoin increased with the maturity of the plant, so that values at the end of flowering (P<0.01), or the green seeds (P<0.001), were higher than for the start of flowering.

The RDA assay indicated that the biological activity of CT of leaves was higher at the start of flowering compared to the end of flowering (P<0.001), as well as for stems (P<0.01) and whole plant (P<0.001) (Table 2). The CT content of leaves, stems and whole plant was higher at the start of flowering compared to the end of flowering (P<0.01). Sainfoin (whole plant) at the end of flowering had a PD:PC ratio (*i.e.,* PD value) higher (P<0.01) than the green seeds and similar to the start of flowering. The *cis:trans* ratio (*i.e*., *cis* value) of leaf, stem or whole plant CT was similar between the start and the end of flowering. The mDP of CT in the whole plant decreased (P<0.001) progressively from the end of flowering, to the green seeds and start of flowering stages.

3.2. In vivo digestibility and nitrogen balance

Organic matter digestibility (OMD) was higher at the start of flowering than for the other phenological stages (Table 3) and was affected by PEG addition at the end of flowering.

Total tract N digestibility increased with PEG addition at each of the three phenological stages. PEG inactivated the tannins and decreased faecal N excretion (P<0.001). Thus, in the presence of active tannins (without PEG), urinary N expressed as g/g N intake was lower (P<0.05) than with PEG addition (Table 3). N retention in g/g N intake was higher (P<0.001) at the start of flowering than at the end of flowering. However, the amount of N retained (expressed in g/g N intake or in g/d/sheep) was not affected by PEG.

3.3. Rumen fluid composition

Whatever the vegetation cycle and the phenological stage, the N and NH_3-N contents in the rumen fluid peaked at 1.5 h and then declined up to 6 h after feeding. The mean N in the rumen fluid increased (P<0.05), as well as the NH_3-N content (P<0.01), by PEG addition (Table 4).

PEG addition affected VFA content in the rumen fluid at the green seeds stage (P<0.05), whereas the total number of protozoa and the number of protozoa smaller than 100μm were not affected by PEG.

3.4. In situ dry matter and nitrogen disappearance in the rumen

The leaf DM disappearance (DisDM), was increased by PEG addition (P<0.001) (Table 5). Likewise, DisN increased (P<0.001) with PEG by + 0.15, + 0.08 and + 0.11, respectively, for the end of flowering, green seeds and start of flowering stages. DisN of leaves was higher at the end of flowering than at the green seeds stage (P<0.001).

PEG increased DisDM (P<0.05) and DisN (P<0.001) of stems (Table 6). Also, DisDM of stems was higher (P<0.001) at start of flowering than at its end or green seeds. Whilst the DisDM of stems was lower than that of leaves, the DisN of stems was higher than that of leaves.

3.5. In situ nitrogen digestion in the small intestine

The N remaining in the leaf residues after 16 h of incubation in the rumen (expressed in g/g initial forage N or in g/kg initial forage DM) was lower with PEG addition, with the effect of PEG more pronounced at the start of flowering (T×Phs interaction, P<0.001, Table 7). For leaves, PEG addition decreased the amount of N disappearing in the small intestine whilst raised the N digestibility in the small intestine (P<0.05).

The N remaining in the stem residues after 16 h of incubation in the rumen was reduced also by PEG addition. A different behaviour between leaf and stem N was observed in the small intestine in that PEG had no effect on the amount of N in stem that disappeared in the SI (Table 7). N

digestibility in the small intestine was increased by PEG more markedly for the end of flowering (T×Phs interaction, $P<0.001$).

4. Discussion

We explored how condensed tannins differ among sainfoin leaves, stems and the whole plant, and how these differences relate to digestion in sheep. We focus on the phenological stages of end of flowering and start of flowering as they are mainly used in practice as feedstuffs. The nutritional effects of CT in forages were assessed by administrating PEG to the rumen of one group of sheep while a separate group received water to allow determination of CT effects by comparing the CT-acting group with the PEG group (Min et al., 2003).

4.1. Characterisation of sainfoin condensed tannins

Despite several studies on CT contents and structures of *Lotus* species (Foo et al., 1982; Hedqvist et al., 2000; Min et al., 2003; Meagher et al., 2004) there are only a few reports that characterize sainfoin CT. Barry and McNabb (1999) advised that dietary concentrations of <50 CTs g /kg DM would be beneficial for animal health and nutrition. However, these recommendations originated mainly from feeding studies with *Lotus* species and may not be applicable to other feeds (Waghorn and McNabb, 2003; Mueller-Harvey, 2006). In our study, the CT content in the whole plant never exceeded 14 g/kg DM (Table 2). The higher CT content of leaves than stems agree with observations on *Lotus* species (Gebrehiwot et al., 2002). The increase in CT biological activity at the start of flowering was related to the increase in the proportion of leaves in the whole plant, as sainfoin CT are located mainly in the leaves (Lees et al., 1995). In addition, it is known that the content and composition of sainfoin CT changes with leaf age (Koupai-Abyazani et al., 1993) and their ability to bind protein tends to be higher in less mature forage plants (Bate-Smith, 1973).

CT are polymers of different flavanol units. Different combinations of OH and H groups in these monomeric units lead to different classes of polymers, such as prodelphinidins (PD) and

procyanidin (PC) (Figure 1). Polymer composition affects CT properties (Frazier et al., 2010; Sarni-Machado et al., 1999) and is also thought to affect how CT influences digestion, and nutritional value, of forages (Meagher et al., 2004; Mueller-Harvey, 2006). For example, a higher PD:PC ratio enhanced the ability of CT to complex with proteins (Aerts et al., 1999). In our study the PD:PC ratios (*i.e.*, PD value) of leaves and stems did not vary much between the start and end of flowering and, as a result, the PD:PC ratios of the whole plants hardly changed. Guimaraes-Beelen et al. (2006) also observed little variation of the PD:PC ratio between phenological stages (*i.e.,* full growth and flowering) on *Mimosa hostilis*. According to Marais et al. (2000), PD:PC ratios in different sainfoin varieties varied from 36:64 to 93:7. In our study, the PD:PC ratio differed markedly at each phenological stage between leaves and stems.

Sainfoin CT structures appear to resemble *L. pedunculatus* CT (Foo et al., 1982; Marais et al., 2000); but the nutritive value of sainfoin is reportedly much higher than *L. pedunculatus* (reviewed by Min et al., 2003). This suggests that the relevant differences in CT structure between sainfoin and *L. pedunculatus* have not yet been identified.

It has been recognised that the biological activity of 'astringency' of CT, which corresponds to their protein binding capacity, is related to the mDP (*i.e.*, the molecular size of the tannins) (Porter and Woodruffe, 1984; Sarni-Marchado et al., 1999; Cheynier, 2005; Frazier et al., 2010). Other studies on forages also found that CT astringency increased with the mDP up to a point, but at the upper end of the molecular weight scale tannins appeared to be less able to interact with proteins, meaning that larger tannins are less astringent (Mangan, 1988; Aerts et al 1999).

In our study, the higher biological activity of the whole plant at the start of flowering compared to the first vegetation cycle may be also related to tannin molecular size: indeed the mDP was lower at the start of flowering, *versus* the more mature phenological stages. This may indicate that smaller tannins have higher biological activity because they are better able to interact with proteins. The biological activity of leaves at the end of flowering was lower than at its start and this may be related

to their higher mDP, suggesting that as the leaf matured, the active sites on tannins for binding with proteins became fewer in number or that tannin molecules became too large to interact effectively with proteins (Makkar et al., 1988; De Freitas and Mateus 2001).

Moreover, for tanniniferous plants, tannin astringency also increases with PD content (Jones et al., 1976; Aerts et al., 1999). In our data, the higher biological activity of leaves than stems appears to be related to the higher PD:PC ratio of leaf CT.

According to De Freitas and Mateus (2001), the *cis:trans* stereochemistry of the monomeric subunits could affect CT interaction with protein. However the literature contains very little information on this topic. In our study the *cis:trans* ratio did not vary between the vegetation cycles and between stems or leaves.

Barry and McNabb (1999) reported that it is still difficult to link all the parameters which characterise CT with their effects on forage digestion. Thus we examined the hypothesis that the higher values for CT content, biological activity, PD:PC ratio and lower mDP model would lead to a larger PEG effect by inhibiting stronger CT-protein complexes.

4.2. Dry matter and N disappearance in the rumen

CT decreased ruminal DisDM and DisN of separated leaves and stems, which was expected, as one of the main characteristics of CT is that they bind dietary proteins in the rumen and thus reduce excessive protein degradation by micro-organisms (Waghorn et al., 1998; Aerts et al., 1999; Turner and Neel, 2003; Mueller-Harvey, 2006).

To calculate DisDM and DisN of the whole plant, we used the proportions of leaves and stems in the whole plant at the three phenological stages. DisDM with or without PEG was, respectively, 0.524 *versus* 0.480 at the start of flowering, 0.463 *versus* 0.390 at the end of flowering and 0.404 *versus* 0.375 at the green seeds stages. DisN with or without PEG was, respectively, 0.640 *versus* 0.573 at the start of flowering, 0.635 *versus* 0.534 at the end of flowering and 0.577 *versus* 0.528 at

the green seeds stages. This decrease of DisDM and DisN by tannins agrees with results of Aufrère et al. (2008), who compared degradability of sainfoin and tannin-free lucerne.

Although leaves had higher N content than stems, the DisN of leaves was lower than the DisN of stems in both vegetation cycles. This could be interpreted as a consequence of the higher CT content, biological activity and PD:PC ratio in leaves which increased CT protein complexation. However, other factors may account for these observations, as comparable differences in DisN between leaves and stems also occur in lucerne, which has no tannins (Tremblay et al., 2002).

The difference between the DisN values with and without PEG represents the effects of CT. For the stems and leaves, differences between DisN with and without PEG, for all phenological stages, was in accordance with the trend of mDP values. The smaller PEG effect on stems was in accordance with their lower CT content and biological activity. However, the less marked CT effect on DisN for leaves, stems and whole plant at the start of flowering compared with the end of flowering was unexpected. One explanation might be that there was an insufficient quantity of PEG to bind all tannins at the start of flowering stage. In the present study, 60 g of PEG 4000 was infused to the rumen given each day to the sheep to inactivate the tannins. This dose was used to avoid an increase in the passage rate from the rumen, as it is known that PEG often causes diarrhoea (Schiller et al., 1988), but it is quite low compared levels used in other studies on sainfoin (Scharenberg et al., 2007: 100 g/d of PEG 4000, Bermingham et al., 2001: 160 g/d of PEG 3500), on sulla (*Hedysarum coronarium*) (Bermingham et al., 2001: 120 g/d of PEG 3500) or on *L. pedunculatus* (Barry and Manley, 1985: 75 to 100 g/d of PEG 3350). In contrast, there are several studies with other tanniniferous plants that used even less PEG than in our study (Ben Salem, 1998: 20 g/d PEG 4000 on *Acacia cyanophylla L.*; Priolo et al., 2000: 40 g/d PEG 4000 on carob pulp; Decandia et al., 2000: 50 g/d PEG 4000 on *Pistacia lentiscus L.*).

In the present study, sainfoin tannins decreased soluble N and NH_3-N content in the rumen fluid, as shown previously in sheep (Waghorn et al., 1990) and in an *in vitro* continuous culture

system (Dahlberg et al., 1988). This is also in agreement with results obtained for lambs fed with sulla (*Hedysarum coronarium*) (Douglas et al., 1999).

4.3. In situ nitrogen digestion in the small intestine

Beneficial effects of CT-containing forages are associated with a lower ruminal degradation of plant protein and an enhanced flow of undegraded dietary protein to the intestine (Aerts et al., 1999).

The review by Frutos et al. (2004) summarised several studies that reported negative effects of tannins on nutrient absorption from the small intestine. This could be due either to the persistence in the intestine of tannin-protein complexes which failed to dissociate in the abomasum, or tannins complexing with digestive enzymes or endogenous protein (Kariuki and Norton, 2008), or a change in intestinal nutrient absorption caused by tannins interacting with intestinal mucosa.

This decrease in intestinal N digestibility of leaves and stems by CT agrees with other findings in sainfoin, sulla (*H. coronarium*) and *L. pedunculatus* (Waghorn et al., 1994; Bermingham et al., 2001). However, the reduced rumen degradation of leaf protein by CT was balanced by an increase in N digestion in the small intestine, whether of the results were expressed as g N/kg initial forage DM or g/g initial forage N, as the amount of N entering the intestine was enhanced (Table 7).

Although sainfoin leaf CT had a similar structure as *L. pedunculatus* CT in terms of PD:PC ratio (70:30), their effect on intestinal absorption differed. *In vivo* measurements showed that CT in *L. pedunculatus* markedly reduced, or had no effect, on apparent digestibility in the small intestine of sheep (Waghorn et al., 1994; Min et al., 2003). However in our study (mobile bags), the CT of sainfoin leaves increased N disappearing from the small intestine.

In contrast, CT did not affect N disappearance of stems in the small intestine. The distinct structural differences between leaf and stem CT (leaf CT contained a higher proportion of PD and have higher mDP than stem CT) may explain the absence of a CT effect on stem N disappearing from the small intestine.

4.4. In vivo digestibility and N retention

Organic matter digestibility (OMD) was affected by PEG addition only at the end of flowering, although values were slightly higher in the presence of active tannins in both vegetation cycles. This could partly be because PEG increases the transit rate from the rumen and can cause diarrhoea at high doses (Schiller et al., 1988), although there was no diarrhoea in our experiment. Our OMD results contrast with Barry and Manley (1986) with sheep fed *L. pedunculatus,* with Barahona et al. (1997) with sheep fed tropical legumes, and with Bermingham et al. (2001) and Scharenberg et al. (2007) with sheep fed sainfoin, all of whom observed higher OMD with PEG. No difference in OMD occurred between lucerne and sainfoin (Aufrère et al., 2008) and between lucerne, *L. corniculatus* and sainfoin (Kraiem et al., 1990), harvested at the early flowering stage.

For the start and end of flowering, OMD values (0.654 *versus* 0.607) matched previous findings for sainfoin fed as fresh forage (INRA, 2007; Aufrère et al., 2008) or as conserved forage (Scharenberg et al., 2007). The OMD of sainfoin was higher than that of lucerne at the same phenological stage (0.65 *versus* 0.60) (INRA, 2007). In the present study, CT decreased N digestibility of sainfoin. This agreed with Stienezen et al. (1996) who found reduced N digestibility in sheep fed sulla (*H. coronarium*) relative to the same diet supplemented with PEG. Our results are also in agreement with those obtained for other CT-containing plants where sheep were fed a mixture of pasture and *L. corniculatus* (Waghorn et al., 1997) or *L. pedunculatus* with perennial ryegrass (Waghorn and Shelton, 1995). The difference between total tract N digestibility measured with and without PEG, as the PD:PC ratio, remained the same between the start and end of flowering, may be due to the higher CT content and biological activity or the lower mean degree of polymerisation at the start of flowering.

The most striking result was that feeding sainfoin had a marked influence on N excretion. The higher faecal N excretion in the presence of active tannins was matched by lower urinary N excretion (Table 3). This shift can be explained by the lower rumen degradability of nitrogenous compounds

and is confirmed by effects of CT on rumen fluid ammonia and total N. These observations are consistent with the results of Aufrère et al. (2008) and Scharenberg et al. (2007).

In terms of ruminant nutrition, it is important to note that the N retention per g of N ingested was not affected by PEG addition. Waghorn and Shelton (1995) obtained a similar result using a mixture of *L. pedunculatus* and *Lolium perenne*, where CT did not alter N retention. This means that the presence of active tannins in sainfoin does not decrease the N value of the forage, despite a reduction in N digestibility. The change of N level with vegetation stage agrees with Egan and Ulyatt (1980), who reported that retained N (g/d) was halved with a late-cut sainfoin compared with an early-cut sainfoin (mean values were 1.3 and 3.5).

Feeding sainfoin to ruminants can, therefore, be used to alter the form of the excreted N and to reduce environmental N pollution because faecal N outputs are considered to be an environmentally less harmful form of N than urinary N (Mueller-Harvey, 2006; Misselbrook et al., 2005). Urinary N is rapidly converted to NH_3 shortly after it is excreted (Varel et al., 1999) and then to volatile nitrous oxides which are potent greenhouse gases (Kebreab et al., 2004) In contrast fecal N is converted to NH_3 at a much slower rate, retain to the soil and contribute to accumulation of soil OM. (Gressley and Armentaro, 2007; Waghorn, 2008).

Conclusions

This study investigated the consequences of variations in CT content and structure on sainfoin DM and N disappearance in the rumen, in the intestine and on *in vivo* digestibility and N retention.

Sainfoin CT in leaves and stems greatly increased the *in situ* estimate of forage N escaping from the rumen but did not negatively impact the amount of N disappearing in the intestine, which is the main site of amino acid absorption by ruminants. These CT hardly affected OMD, and did not affect N retention, which is beneficial in ruminant nutrition. The nutritive value of sainfoin was higher at the start of flowering, as the plant was less mature and had more N and less fibre. The

larger CT effect at the start of flowering appears to be related to the higher biological activity and lower molecular weight of tannins. These properties may contribute to the higher nutritive value at the start of flowering. The PD:PC ratios of CT in whole plant did not vary among the end of flowering and the start of flowering whereas its mDP vary among the phenological stages.

Results demonstrate the potential of using both quantitative and qualitative CT information, in terms of CT contents and structure such as mDP, PD:PC ratio, as an indicator of their biological properties. A better understanding of the relationships between the chemical structure of CT and their reactivity will improve our appreciation of their contribution to the nutritive value of forages.

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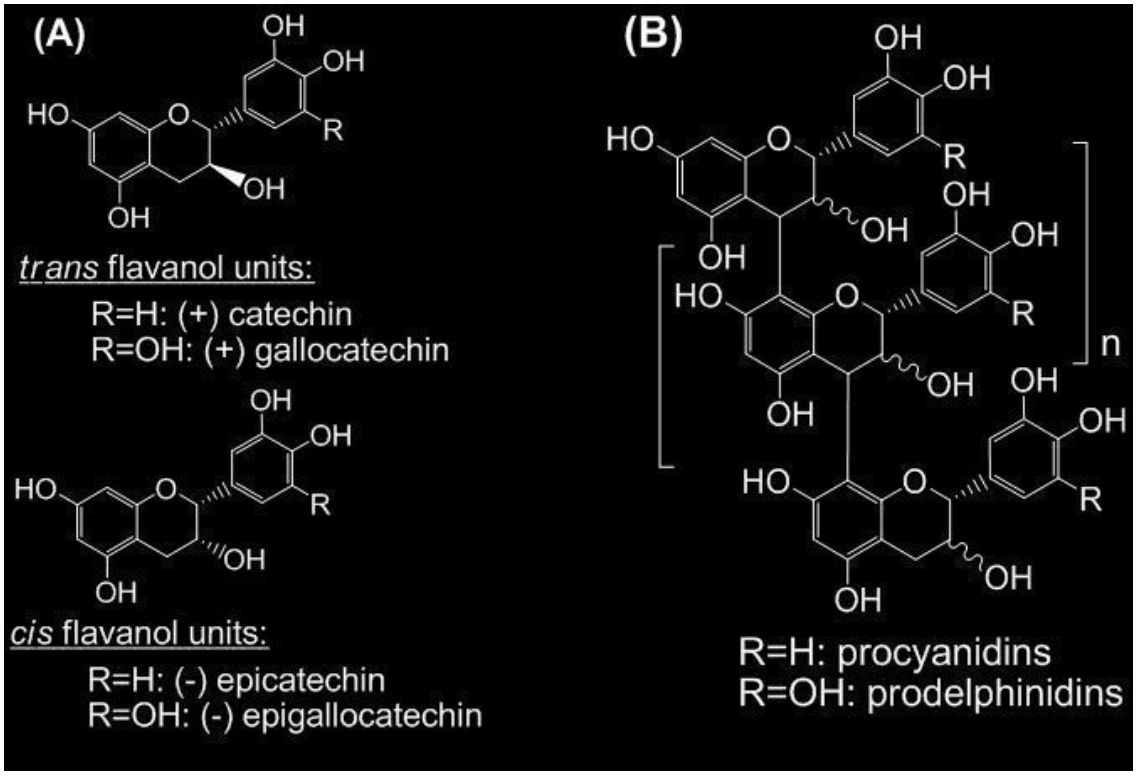

Figure 1. Sainfoin flavanol monomers (A) and condensed tannins (B)

Table 1
Chemical composition (g/kg DM) and pepsin-cellulase digestibility (Dcell) of sainfoin studied as fresh forage at the end of flowering, green seeds and start of flowering.

Phenological stage	First vegetation cycle		Second vegetation cycle	*n*	SEM	Statistical significance		
	End of flowering	Green seeds	Start of flowering			Phs P1 *vs* P2	Phs P1 *vs* P3	Phs P2 *vs* P3
Whole plant (g/kg DM)								
N	22.9	23.2	29.9	3	0.19	*	***	***
aNDF	415	441	355	3	3.5	**	**	***
ADF	314	338	279	3	2.5	**	**	***
Lignin(sa)	67.4	83.0	77.7	3	2.13	**	*	NS
Dcell	557	504	610	3	3.8	***	*	**
Proportion of leaves in the whole plant	0.360	0.350	0.470	3	0.0211	NS	NS	*
Leaves(g/kg DM)								
N	32.7	32.3	37.1	3	0.13	NS	**	***
aNDF	255	296	253	3	0.9	**	NS	**
ADF	215	206	183	3	4.2	**	**	NS
Stems (g/kg DM)								
N	16.1	18.4	22.3	3	0.04	***	***	***
aNDF	526	550	412	3	2.6	*	***	***
ADF	408	431	322	3	2.1	*	***	***

First vegetation cycle: End of flowering (P1), Green seeds (P2), Second vegetation cycle: Start of flowering (P3); NS, not significant ($P>0.05$). Probability of significant effects due to phenological stage (PhS) in the analysis for fresh forages. (*=P< 0.05, **=P<0.01, ***=P<0.001).

Table 2
Characterisation of condensed tannins (CT) of sainfoin studied as fresh forage at the end of flowering, green seeds and start of flowering.

Phenological stage	First vegetation cycle		Second vegetation cycle	*n*	SEM	Statistical significance		
	End of flowering	Green seeds	Start of flowering			Phs P1 *vs* P2	Phs P1 *vs* P3	Phs P2 *vs* P3
CT biological activity (g tannic acid equivalent/100g)(DM)								
Leaves	1.97	2.30	3.40	3	0.180	NS	***	***
Stems	1.00	1.00	1.23	3	0.065	NS	**	*
Whole plant	1.02	1.07	1.58	3	0.049	NS	***	**
CT content (g/kg DM)								
Leaves	5.0	5.3	9.8	3	0.68	NS	**	**
Stems	4.3	3.06	9.0	3	0.64	NS	**	**
Whole plant	6.2	8.3	13.6	3	0.71	NS	**	NS
CT mean degree of polymerisation (mDP)								
Leaves	37.4	27.4	11.8	3	1.67	*	***	**
Stems	24.6	9.4	8.2	3	0.64	***	***	NS
Whole plant	31.0	21.9	11.0	3	0.39	***	***	***
CT prodelphinidin (PD)								
Leaves	80.4	69.1	76.4	3	0.46	***	**	***
Stems	54.1	35.2	55.4	3	0.55	***	NS	***
Whole plant	69.2	66.2	70.5	3	0.53	*	NS	**
CT cis								
Leaves	64.6	71.2	72.8	3	2.47	NS	NS	NS
Stems	84.4	84.8	83.1	3	0.51	NS	NS	NS
Whole plant	82.9	79.0	81.7	3	0.58	**	NS	*

First vegetation cycle: End of flowering (P1), Green seeds (P2), Second vegetation cycle: Start of flowering (P3); NS, not significant ($P>0.05$). Probability of significant effects due to phenological stage (Phs) in the analysis for fresh forages. (*=P< 0.05, **=P<0.01, ***=P<0.001).

Table 3
Total tract organic matter (OM) and nitrogen (N) digestibility, N losses and N retention for sheep fed fresh sainfoin at the end of flowering, green seeds or start of flowering with or without addition of polyethylene glycol (PEG) in the rumen.

Phenological stage	First vegetation cycle				Second vegetation cycle		SEM	T	Statistical significance						
	End of flowering		Green seeds		Start of flowering				Phs P1 *vs* P2	Phs P1 *vs* P3	Phs P2 *vs* P3	T×P1	T×P2	T×P3	T×Phs
PEG treatment	with	without	with	without	with	without									
N intake (g/d/sheep)	29.86	28.29	40.2	37.20	42.8	41.51	1.040	NS	***	***	***	NS	NS	NS	NS
Digestibility															
OM	0.581	0.607	0.519	0.558	0.648	0.654	0.0115	NS	**	***	***	**	NS	NS	*
N (total tract)	0.677	0.601	0.654	0.590	0.775	0.665	0.0086	***	NS	***	***	***	**	***	***
N (g/g N intake)															
Faeces	0.319	0.399	0.346	0.409	0.224	0.336	0.0129	***	NS	***	***	***	**	***	NS
Urine	0.454	0.410	0.398	0.330	0.478	0.370	0.0286	*	*	NS	*	NS	NS	*	NS
Nitrogen retained															
g/d/ sheep	6.77	5.49	10.22	9.99	12.72	12.30	1.146	NS	**	***	*	NS	NS	NS	NS
g/g N intake	0.23	0.19	0.25	0.26	0.30	0.29	0.029	NS	NS	***	NS	NS	NS	NS	NS

First vegetation cycle: End of flowering (P1), Green seeds (P2), Second vegetation cycle: Start of flowering (P3). NS, not significant ($P>0.05$). Probability of significant effects due to treatment (T), phenological stage (Phs) and their interaction (T×Phs) in the analysis for fresh forages (*=P< 0.05, **=P<0.01, ***=P<0.001).

Table 4
Concentration of Nt (mg/g) and NH_3-N (mg/g), volatile fatty acids (mmol/L) and protozoa in the rumen fluid of sheep fed sainfoin as fresh forage at the first vegetation cycle (end of flowering and green seeds) and at the second vegetation cycle (start of flowering) with or without addition of polyethylene glycol (PEG) in the rumen.

Phenological stage	First vegetation cycle				Second vegetation cycle		SEM	T	Statistical significance							
	End of flowering		Green seeds		Start of flowering				Time	Phs P1 vs P2	Phs P1 vs P3	Phs P2 vs P3	T×P1	T×P2	T×P3	T×Phs
PEG treatment	with	without	with	without	with	without										
Total N	0.23	0.19	0.24	0.21	0.28	0.22	0.008	*	***	**	***	NS	**	*	***	NS
Ammonia N (NH_3-N)	0.20	0.17	0.23	0.20	0.26	0.21	0.004	**	***	***	***	***	**	**	***	NS
Volatile fatty acids (VFA)	92.0	83.6	91.8	100.9	96.8	89.5	3.04	NS	***	***	*	NS	NS	*	NS	***
Acetate:propionate ratio	3.35	3.58	3.67	4.19	4.08	3.72	0.230	NS	***	*	*	NS	NS	NS	NS	NS
Protozoa (x 10^5/ml)																
<100µm	1.83	1.61	1.35	1.51	2.21	2.10	0.241	NS	***	NS	**	***	NS	NS	NS	NS
>100µm	0.08	0.01	0.03	0.01	0.02	0.03	0.008	**	***	**	**	NS	***	NS	NS	***
Total	1.91	1.62	1.38	1.52	2.24	2.13	0.244	NS	***	*	*	***	NS	NS	NS	NS

First vegetation cycle: End of flowering (P1), Green seeds (P2), Second vegetation cycle: Start of flowering (P3). NS, not significant ($P>0.05$). Probability of significant effects due to treatment (T), phenological stage (Phs) and their interaction (T×Phs) in the analysis for fresh forages (*=P< 0.05, **=P<0.01, ***=P<0.001).

Table 5
In situ rumen disappearance (Dis) of dry matter and N of the leaves of sainfoin studied as fresh forage at the first vegetation cycle (end of flowering and green seeds) and at the second vegetation cycle (start of flowering) with or without addition of polyethylene glycol (PEG) in the rumen.

Phenological stage	First vegetation cycle				Second vegetation cycle		SEM	Statistical significance							
	End of flowering		Green seeds		Start of flowering			T	Phs P1 vs P2	Phs P1 vs P3	Phs P2 vs P3	T×P1	T×P2	T×P3	T×Phs
PEG treatment	with	without	with	without	with	without									
Dry matter disappearance															
a	0.056	0.095	0.086	0.107	0.049	0.084	0.0087	***	*	NS	*	**	NS	NS	NS
b	0.757	0.768	0.783	0.751	0.837	0.834	0.0114	NS	NS	***	***	NS	NS	NS	NS
c	0.195	0.068	0.083	0.066	0.103	0.062	0.0150	***	**	**	NS	***	NS	NS	**
DisDM	0.616	0.503	0.539	0.491	0.577	0.507	0.0116	***	**	NS	NS	***	**	***	NS
Nitrogen disappearance															
a	0.054	0.064	0.069	0.083	0.044	0.065	0.0098	*	NS	NS	NS	NS	NS	NS	NS
b	0.858	0.893	0.890	0.888	0.937	0.955	0.0132	NS	NS	***	***	NS	NS	NS	NS
c	0.176	0.063	0.078	0.052	0.100	0.056	0.0121	***	***	**	NS	***	NS	*	**
DisN	0.675	0.523	0.571	0.490	0.628	0.523	0.0112	***	***	NS	**	***	***	***	*

First vegetation cycle: End of flowering (P1), Green seeds (P2), Second vegetation cycle: Start of flowering (P3). 'a': rapidly disappearing fraction, 'b': slowly disappearing fraction, 'c': disappearance rate of fraction b (h^{-1}), DisDM or DisN: *in situ* ruminal disappearance of DM or N: *in situ* ruminal disappearance of DM or N calculated with a passage rate of 0.06/h; NS, not significant ($P>0.05$). Probability of significant effects due to treatment (T), phenological stage (Phs) and their interaction (T×Phs) in the analysis for fresh forages (*=P< 0.05, **=P<0.01, ***=P<0.001).

Table 6
In situ rumen disappearance (Deg) of dry matter and N of the stems of sainfoin studied as fresh forage at the first vegetation cycle (end of flowering and green seeds) and at the second vegetation cycle (start of flowering occurred 5 weeks after the end of flowering of the first vegetation cycle) with or without addition of polyethylene glycol (PEG) in the rumen.

	First vegetation cycle				Second vegetation cycle			Statistical significance							
Phenological stage	End of flowering		Green seeds		Start of flowering		SEM	T	Phs P1 vs P2	Phs P1 vs P3	Phs P2 vs P3	T×P1	T×P2	T×P3	T×Phs
PEG treatment	with	without	with	without	with	without									
Dry matter degradability															
a	0.054	0.067	0.062	0.061	0.062	0.077	0.0064	NS	NS	NS	NS	NS	NS	NS	NS
b	0.529	0.557	0.519	0.508	0.682	0.667	0.0161	NS	NS	***	***	NS	NS	NS	NS
c	0.102	0.055	0.066	0.061	0.093	0.081	0.0085	*	NS	NS	**	***	NS	NS	*
DisDM	0.377	0.326	0.332	0.313	0.477	0.457	0.0086	**	***	***	***	***	NS	NS	NS
Nitrogen degradability															
a	0.058	0.078	0.082	0.097	0.071	0.0.68	0.0103	NS	*	NS	*	NS	NS	NS	NS
b	0.785	0.741	0.748	0.716	0.869	0.872	0.0146	NS	**	***	***	*	NS	NS	NS
c	0.152	0.113	0.121	0.104	0.120	0.090	0.0129	NS	NS	NS	NS	*	NS	NS	NS
DisN	0.612	0.540	0.580	0.549	0.650	0.617	0.0085	***	NS	***	***	***	NS	NS	NS

First vegetation cycle: End of flowering (P1), Green seeds (P2), Second vegetation cycle: Start of flowering (P3). 'a': rapidly disappearing fraction, 'b': slowly disappearing fraction, 'c': disappearance rate of fraction b (h^{-1}), DisDM or DisN: *in situ* ruminal disappearance of DM or N: *in situ* ruminal disappearance of DM or N calculated with a passage rate of 0.06/h; NS, not significant ($P>0.05$). Probability of significant effects due to treatment (T), phenological stage (Phs) and their interaction (T×Phs) in the analysis for (T×Phs) in the analysis for fresh forages (*=P< 0.05, **=P<0.01, ***=P<0.001).

Table 7
N remaining in bags after 16 h incubation in the rumen of leaves and of stems of fresh sainfoin, forage N disappearing from mobile nylon bags in the SI and disappearance in the SI of 16 h rumen residual forage N. All results were expressed in g initial forage N and in g/kg initial forage DM, except digestibility in SI.

Phenological stage	First vegetation cycle				Second vegetation cycle		SEM	Statistical significance							
	End of flowering		Green seeds		Start of flowering			T	Phs P1 vs P2	Phs P1 vs P3	Phs P2 vs P3	T×P1	T×P2	T×P3	T×Phs
PEG Treatment	with	without	with	without	with	without									
Leaves															
N remaining after 16 h in the rumen bags															
g/g initial forage N	0.25	0.46	0.38	0.45	0.28	0.51	0.024	***	*	NS	NS	***	NS	***	**
g/kg initial forage DM	8.26	14.97	12.30	14.45	10.53	18.80	0.831	***	NS	*	NS	***	NS	***	***
N disappearing in SI															
g/g initial forage N	0.14	0.22	0.21	0.22	0.15	0.24	0.026	*	NS	NS	NS	*	NS	*	NS
g/kg initial forage DM	4.47	7.13	6.51	6.95	5.74	8.90	0.866	*	NS	NS	NS	**	NS	**	NS
Digestibility in SI	0.50	0.48	0.55	0.48	0.54	0.47	0.029	*	NS	NS	NS	NS	NS	*	NS
Stems															
N remaining after 16h in the rumen bags															
g/g initial forage N	0.32	0.43	0.39	0.41	0.21	0.30	0.009	***	*	***	***	***	NS	***	***
g/kg initial forage DM	5.19	6.85	7.11	7.48	4.73	6.77	0.151	***	***	NS	***	***	*	***	***
N disappearing in SI															
g/g initial forage N	0.11	0.10	0.19	0.14	0.10	0.11	0.024	NS	*	NS	*	NS	NS	NS	NS
g/kg initial forage DM	1.82	1.66	3.55	2.61	2.24	2.46	0.465	NS	***	NS	NS	NS	NS	NS	NS
Digestibility in SI	0.37	0.16	0.46	0.39	0.36	0.32	0.034	**	***	**	*	***	NS	NS	*

First vegetation cycle: End of flowering (P1), Green seeds (P2), Second vegetation cycle: Start of flowering (P3). Not significant ($P>0.05$). Probability of significant effects due to treatment (T), phenological stage (Phs) and their interaction (T×Phs) in the analysis for fresh forages (*=P< 0.05, **=P<0.01, ***=P<0.001).

PART 2

Effects of condensed tannins of sainfoin on the production and composition of fermentation gases

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In vitro **study of the effects of condensed tannins in sainfoin on the digestive process in the rumen at two vegetation cycles.**

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Abstract

One approach to reducing the production of greenhouse gases is to use tanniniferous forage plants such as sainfoin. The objective of this study was to investigate, *in vitro*, the effects of condensed tannins (CT) in the whole plant, leaves and stems of sainfoin (*Onobrychis viciifolia*) on the digestive process in the rumen. Sainfoin, studied during the first vegetation cycle at the end of flowering, and during the second vegetation cycle at the start of flowering and was incubated in batches containing buffered rumen fluid for 3.5 h and 24 h. To assess the effects of CT, measurements were made with and without added polyethylene glycol (PEG), which neutralises CT effects. Biological activity and prodelphinidin (PD) content were higher (P<0.001) in leaves than in stems, but *cis* value was lower (P<0.01). At 3.5 h of incubation gas production was greater for stems than for leaves (P<0.05), but at 24 h it was higher for leaves than for stems (P= 0.096) at the end of flowering. The presence of PEG at both short and long fermentation times increased gas production of leaves, stems and the whole plant (P<0.001). At 3.5 h, PEG addition resulted in higher CH_4 values for stems and leaves at two vegetation cycles (P<0.01). The CO_2:CH_4 ratio was lower (P<0.01) at 3.5 h and higher at 24 h (P<0.01) with the added PEG. At both 3.5 h and 24 h, values of the CH_4:TDOM ratio were higher for stems than for leaves (P<0.001), and added PEG resulted in a higher ratio (P<0.001) for both stems and leaves. No significant effect of PEG on the whole plant was observed for CH_4 production or the CO_2:CH_4 ratio at either 3.5 h or 24 h incubation. At both incubation periods, *in vitro* true organic matter digestibility (IVTOMD) was higher for leaves than for stems (P<0.001) and decreased with added PEG at 24 h (P<0.001). For the whole plant, treatment with PEG had no significant IVTOMD effect at either 3.5 h or 24 h of

incubation. At 3.5 h, added PEG resulted in a higher nitrogen disappearance (ND) at both vegetation cycles ($P<0.01$), whereas the values were higher at the second one ($P<0.001$). Mean volatile fatty acid production was higher for leaves than for stems ($P<0.01$) and increased with added PEG ($P<0.001$) for both vegetation cycles at 3.5 h and 24 h of incubation. For the two incubation periods PEG increased ($P<0.001$) NH_3-N for leaves and stems at both vegetation cycles. For the whole plant, added PEG resulted in higher NH_3-N content ($P<0.001$) at both 3.5 h and 24 h of incubation.

Abbreviations: ADF, acid detergent fibre expressed inclusive of residual ash; aNDF, neutral detergent fibre assayed with a heat-stable amylase and expressed inclusive of residual ash; BSA, bovine serum albumin; BW, body weight; $BW^{0.75}$, metabolic BW; CT, condensed tannins; CH_4, methane; Dcell, pepsin-cellulase digestibility; DM, dry matter; GP, gas production; HT, hydrolysable tannins; IVTOMD, *in vitro* true organic matter digestibility; mDP, mean degree of polymerisation; N, nitrogen; ND; N disappearance; NH_3-N, ammonia N; OM, organic matter; OMD, OM digestibility; PC, procyanidins; PD, prodelphinidins; PEG, polyethylene glycol; PF, partitioning factor; PP, part of the plant; RDA, radial diffusion assay; sa, sulphuric acid; T, treatment; TDOM, truly digestible organic matter, VC, vegetation cycle; VFA, volatile fatty acids.

Keywords: Sainfoin, *In vitro* gas production, Tannins, Polyethylene glycol, Methane, Ruminal fermentation.

1. Introduction

Ruminants are important producers of greenhouse gases including methane (CH_4) and nitrous oxide (N_2O). The production of greenhouse gases from livestock and their impact on climate change are a major concern worldwide (Steinfeld *et al*., 2006). One possible approach to reducing these emissions might be to use tanniniferous forage plants such as sainfoin in animal nutrition. Several studies have demonstrated a pronounced shift in N excretion from urine to faeces (Mueller-Harvey, 2006; Scharenberg et al. 2007; Aufrère et al. 2008; Theodoridou et al. 2010) and also a decrease in CH_4 emission by ruminants consuming CT-containing forage (Waghorn et al., 2002; Tavendale et al., 2005; Puchala *et al*., 2005; Martin et al., 2009).

Sainfoin is a temperate forage that contains condensed tannins (CT), which are able to bind protein and reduce their degradation in the rumen (Min et al., 2003, Waghorn, 2008). However, there have been few studies on gas production from sainfoin. There is also insufficient knowledge on the processes that account for the effect of tanniniferous forages on digestion in relation to plant parts (stems and leaves) and CT physical and chemical structure. Investigation of the effect of CT of different plant parts on digestion is of obvious interest as leaves are observed to have higher CT content, biological activity and PD:PC ratio (Theodoridou et al., 2010), increasing CT protein complexation (Jones et al., 1976; Aerts et al., 1999).

The aim of this study was to investigate, *in vitro*, the effects of condensed tannins in whole plant, leaves and stems of sainfoin (*Onobrychis viciifolia*) on the digestive process in the rumen at two vegetation cycles. To assess effects of sainfoin CT, measurements

were made with and without added polyethylene glycol (PEG), which deactivates CT (Waghorn and Shelton, 1997; Barry et al., 2001; Makkar, 2003).

2. Materials and Methods

2.1 Forages

Sainfoin (*Onobrychis viciifolia*) cv. Perly was grown in Clermont-Ferrand (France), at INRA's Crouël site at an altitude of 320 m on a fertile, basic, loamy soil (374 g/kg sand, 458 g/kg loam, 168 g/kg clay), 37 g/kg organic matter (OM) of pH 8.2. No mineral fertiliser was applied. Experiments were conducted during 2008 with fresh forages harvested during the first vegetation cycle at the end of flowering (*i.e.*, half of the flower stems showed open flowers on their upper halves) and during the second vegetation cycle at the start of flowering (*i.e.*, half of the flower stems showed open flowers on their lower halves). First vegetation cycle from 2 to 11 June and second vegetation cycle from 7 to 16 July, (*i.e.*, five weeks after the first vegetation cycle). For each vegetation cycle, after harvesting the whole sainfoin plant, stems and leaves were separated manually; one part (300 g) was dried at 60°C for 48 h for chemical analysis, and the rest was freeze-dried (300 g) for CT analysis and *in vitro* experiments.

2.2 Experimental design

Experiments were conducted in triplicate to measure *in vitro* rumen fermentation characteristics of sainfoin and to determine the effect of vegetation cycle and different parts of the plant by the presence of CT.

Two incubation periods were investigated: 3.5 h corresponded to the fermentation of the soluble, fast-fermentable fraction, including water-soluble carbohydrates (WSC), and

microbial protein synthesis, and 24 h to the fermentation of the non-soluble fraction and before its bacteriolysis. For each incubation period, total gas production (GP) and *in vitro* true organic matter digestibility (IVTOMD) were determined to calculate the partitioning factor (PF) (TDOM/gas produced) as described by Blümmel et al. (1997). The CH_4:TDOM ratio, an indicator of the efficiency of digestion, was also calculated. Gas composition (CH_4, CO_2), volatile fatty acids (VFA) and ammonia nitrogen (NH_3-N) contents in the medium, and nitrogen (N) disappearances (ND) were also determined. The effects of CT were determined by adding polyethylene glycol (PEG).

2.3 In vitro batch fermentations and sampling

Three castrated male Texel sheep (12 months old, 60 ± 3.2 kg body weight (BW)) fitted with rumen cannulae were used as rumen fluid donors. The experimental period comprised two weeks for adaptation, when sheep were offered 1200 g daily of a diet comprising, per kg, 800 g cocksfoot hay, 60 g barley, 50 g pulp beet; 28 g soya bean meal, 34 g corn, 12 g wheat and 16 g molasses and minerals, in two equal meals per day at 08:00 h and 16:00 h. Each sample of the three substrates (leaves, stems and whole plant, at the two vegetation cycles) was weighed (600 mg) in duplicate (n=24) and incubated in 120 mL serum bottles (Wheaton, Millville, NJ). The same procedure was followed for the treatment with added PEG on further samples of the three substrates (n=12). The quantity of PEG used was 2.3g/l of phosphate:carbonate buffer solution. For each batch the following procedure was used. Rumen digesta were collected from each of the three sheep before the morning feeding, mixed in equal proportions, transported to the laboratory and then squeezed through a polyester monofilament fabric (mesh opening 800 µm). Rumen fluid was diluted 1:2 (vol/vol) in the anaerobic phosphate:carbonate buffer

solution described by Goering and Van Soest (1970) modified by Niderkon et al. (2010) and with the following modification: no NH_4HCO_3 was used in the preparation of the bicarbonate buffer, which consisted entirely of $NaHCO_3$ to optimize the use of N from the substrate by rumen microbes. Aliquots (40 mL) of buffered rumen fluid were dispensed into serum bottles previously warmed to 39°C and flushed with N_2. The bottles were sealed with butyl rubber stoppers (Bellco Glass Inc., Vineland, NJ) and aluminium crimp seals, and incubated for 3.5 h or 24 h in a shaking water bath at 39°C. The contents of the fermentation bottles were stirred intermittently using a magnetic bar (300 rpm for 30 s and then left to rest for 2.5 min). In each batch a blank of buffered rumen fluid without sample was incubated.

The gas pressure in the serum bottles was recorded at 0, 0.75, 2, 3.5, 7, 9 and 24 h of incubation using the pressure transducer technique, as described by Theodorou et al. (1994). For the two incubation periods (3.5 h and 24 h) 48 samples were used for each treatment with or without added PEG. For each treatment, incubation of one half of the samples ended at 3.5 h and incubation of the other half ended at 24 h. At the end of each incubation period the gas in the headspace was sampled for analysis of composition. The entire contents of each serum bottle was transferred to a pre-weighed Falcon™ tube, the pH was immediately measured, and the tubes were centrifuged at 3,400 $\times$ *g* for 10 min. An aliquot of supernatant (5 mL) was transferred to a polypropylene tube containing 0.5 mL of H_3PO_4 5% (vol/vol) and frozen at −20°C for NH_3-N analysis. A second aliquot of supernatant (0.8 mL) was transferred to a microtube containing 0.5 mL of deproteinising solution (crotonic acid 0.4% wt/vol, metaphosphoric acid 2% wt/vol, in HCl 0.5M). The mixture was cooled at 4°C for 2 h, centrifuged at 16,500 $\times$ *g* for 10 min

at 4°C, and the supernatant was frozen at −20°C for VFA analysis. The serum bottle was washed twice with distilled water to recover all the non-degraded particles, which were transferred into the Falcon™ tube. The tubes were centrifuged at 3,400 $\times$ *g* for 10 min, the supernatant was removed and the residue was stored at −18°C for determination of OM, neutral detergent fibre (aNDF) and N. The whole process was realised in three replicates.

2.4 Laboratory analyses

Nitrogen in the forages (*i.e.,* whole plant, leaves and stems), was determined using a Kjeldahl method according to AOAC (1990, method 977.02). Neutral detergent fibre (aNDF), acid detergent fibre (ADF) and lignin (sa) of the fresh forages, leaves and stems were analysed in filter bags according to Van Soest and Wine (1967) as modified by Pagan et al. (2009). Pepsin-cellulase digestibility of the forages (whole plant) was determined according to Aufrère and Michalet-Doreau (1983). Tannins were analysed by direct thiolysis of freeze-dried samples with benzyl mercaptan at 40°C for 1 h to obtain quantitative and qualitative information on tannins (Guyot et al., 2001; Gu et al., 2003). Quantitative data referred to the content of extractable plus unextractable CT, while qualitative data provided information on tannin structures, such as mean degree of polymerisation (mDP) (*i.e.,* molecular size), prodelphinidin:procyanidin (PD:PC) ratio (PD value) and *cis*:*trans* ratio (*cis* value). The biological activity of CT in terms of protein-binding capacity was determined using the radial diffusion assay (RDA; Makkar et al., 2000). This assay measures precipitation of bovine serum albumin (BSA) by tannins, the area of the precipitation ring being proportional to the amount of extracted CT. For the RDA assay, CT were extracted with acetone-water (7:3, v/v) from freeze-

dried leaves, stems or whole plant. Individual and total VFA concentrations were analysed using gas liquid chromatography as described by Jouany (1982), and NH_3-N was measured using the Berthelot reaction (Weatherburn 1967). The composition of fermentation gases was determined by GLC as described by Macheboeuf et al. (2008). NDF content in residues of fermentation was determined using an autoclave in neutral detergent solution at 110°C for 40 min as recommended by Senger et al. (2008). This procedure allows removal of solid-associated bacteria in the residue and thus calculation of *in vitro* true organic matter digestibility (IVTOMD).

2.5 Calculations and statistical analysis

The blanks were used to calculate the amounts of plant components degraded and the net production of fermentation end-products at the end of each incubation period.

Data were analysed using the SAS Mixed Procedure (2000). The model used to evaluate the effect of CT in the whole plant included vegetation cycle (VC), treatment (T) (with or without PEG) and the interaction VC*T as fixed effects, and replication as a random effect.

The effect of CT of different plant parts (leaves or stems) was evaluated according to a statistical model including vegetation cycle (VC), treatment (T) (with or without PEG), plant part (nested to the vegetation cycle) and the interactions VC*T and T*PP as fixed effects, and replication as a random effect.

3. Results

3.1 Chemical composition of the forages

The N content of the whole sainfoin plant was higher (P<0.01) at the first than at the second vegetation cycle (Table 1). Nitrogen and PD (P<0.001) and CT biological activity (P<0.001) were higher for leaves than for stems. The mDP value and CT content did not vary among parts of the plant, but the *cis* value was higher (P<0.01) for stems. CT biological activity and N content of leaves, stems and whole plant was higher at the second vegetation cycle (P<0.001). aNDF and ADF content were lower for leaves than for stems (P<0.001) at two vegetation cycles, and the values were lower for the less mature plants at the second vegetation cycle (P<0.001).

Sainfoin (whole plant) at the end of flowering had a PD:PC ratio (*i.e.,* PD value) similar to that at the second vegetation cycle. The *cis:trans* ratio (*i.e*., *cis* value) of leaf, stem or whole plant CT was similar at both two vegetation cycles. The mDP of CT in leaves, stems and whole plant was higher at the end of flowering (P<0.01).

3.2 In vitro gas production

Stems and leaves

The total volume of gas produced at 3.5 h of incubation (Table 3) was greater for stems than for leaves without added PEG (P<0.05). The effect of CT was greater for leaves than for stems (P<0.001).

By contrast, total gas production at 24 h tended to be higher for leaves than for stems (P= 0.096) at the first vegetation cycle. At the second vegetation cycle the higher values were obtained for stems (P=0.001) (Table 4). There was a considerable increase in gas

production when stems or leaves were incubated in the presence of PEG at both early and late fermentation ($P < 0.001$).

Whole plant

At 3.5 h and 24 h of incubation (Table 5 and Table 6) the gas production of the whole plant of sainfoin was higher at the start (second vegetation cycle) than at the end of flowering 9first vegetation cycle) ($P < 0.001$). The gas production increased ($P < 0.001$) with added PEG at both fermentation times.

3.3 Methane production

Leaves and stems

Accumulated CH_4 production after 3.5 h (Table 3) incubation was higher for stems than for leaves ($P < 0.01$). The same results were obtained for 24 h of incubation (Table 4). PEG addition resulted in higher CH_4 values for both stems and leaves at both vegetation cycles and fermentation times ($P < 0.01$). At 3.5 h and 24 h the differences between the CH_4 produced with and without PEG were higher at the first than at the second vegetation cycle and higher for leaves than for stems.

At 3.5 h, the CO_2:CH_4 ratio was lower with added PEG ($P<0.01$) irrespective of the plant part at both two vegetation cycles. At 24 h, there was a significant effect of plant part on the CO_2:CH_4 ratio, and higher values were obtained for the stems than for the leaves ($P<0.01$). The same ratio increased with added PEG ($P<0.01$) and was higher at the first vegetation cycle ($P<0.05$).

For 3.5 h and 24 h, values of the CH_4:TDOM ratio were higher for stems than for leaves ($P<0.001$), and added PEG resulted in a higher ratio ($P<0.001$) for both stems and leaves. The mean CO_2:CH_4 ratio was lower at the second vegetation cyle ($P<0.05$).

Whole plant

At 3.5 h and 24 h (Table 5 and Table 6), no significant effect of PEG was observed on either CH_4 production or CO_2:CH_4 ratio. No effect of vegetation cycle was observed for CH_4 production at 3.5 h.

At 3.5 h and 24 h, the ratio of CH_4: TDOM was higher with PEG addition. At 3.5 h at the first vegetation cycle the same ratio was lower than at the second vegetation cycle ($P < 0.05$). The CO_2:CH_4 ratio did not differ between the two vegetation cycles at 24 h.

3.4 Organic matter digestibility, nitrogen disappearance and partitioning factor

Stems and leaves

At 3.5 h and 24 h, (Table 3 and Table 4) the IVTOMD was significantly higher for leaves than for stems ($P < 0.001$) and decreased significantly with added PEG at 24 h. The values for IVTOMD were higher at the second vegetation cycle than the first one ($P < 0.001$) at both 3.5 h and 24 h.

At 3.5 h, nitrogen disappearance (ND) was significantly affected by the different parts of the plant, and the values were slightly higher for stems than for leaves ($P < 0.01$). Addition of PEG resulted in a higher ND at both vegetation cycles ($P < 0.01$), whereas the values were higher at the second vegetation cyle ($P < 0.001$). At 24 h, ND did not differ between leaves and stems, and the values increased with added PEG ($P < 0.01$).

At 3.5 h, the partitioning factor (PF) (TDOM/ ml gas) was higher ($P < 0.001$) for leaves than for stems, and the values were lower at the first vegetation cycle ($P < 0.05$). PEG addition had a significant effect, and decreased PF value ($P < 0.001$) irrespective of the vegetation cycle or part of the plant.

At 24 h, the PF was found to be higher for leaves than for stems ($P < 0.001$) and decreased with added PEG at both two vegetation cycles ($P < 0.001$). Higher values of PF were observed at the second vegetation cycle ($P < 0.001$).

Whole plant

IVTOMD had the highest values at the second vegetation cycle (start of flowering) ($P < 0.001$), whereas treatment with PEG had no significant effect at either 3.5 h or 24 h of incubation (Table 5 and Table 6). ND of the whole sainfoin plant was greater at the second than at the first vegetation cycle ($P < 0.001$) and no differences were observed with added PEG. At both incubation periods the partitioning factor was higher at the second vegetation cycle ($P < 0.05$) in this cycle its value decreased more with added PEG.

3.5 Volatile fatty acids (VFA)

Stems and leaves

The mean VFA production was higher for leaves than for stems ($P < 0.01$), and PEG addition increased VFA ($P < 0.001$) at both vegetation cycles at 3.5 h of incubation (Table 3 and Table 4). At 24 h of incubation added PEG resulted in a decrease in VFA production for stems ($P < 0.001$). The effect of PEG was greater for leaves than for stems at both 3.5 h and 24 h of incubation ($P < 0.001$).

At 3.5 h and 24 h differences were found in the acetate-to-propionate ratio (C2:C3) between stems and leaves, and the values were higher for leaves than for stems ($P < 0.001$) and decreased with added PEG ($P < 0.001$) at both vegetation cycles. At 3.5 h of incubation the mean C2:C3 ratio was higher at the second than at the first vegetation cycle ($P < 0.001$).

Whole plant

At 3.5 h values of VFA were higher at the second than at the first vegetation cycle ($P < 0.001$) and increased with PEG addition ($P < 0.001$). However, the production of VFA at 24 h was not significantly affected by either the vegetation cycle or PEG addition (Table 5 and Table 6).

At 3.5 h or 24 h the addition of PEG did not affect C2:C3 ratio. The mean values were higher at the second vegetation cycle ($P < 0.001$) only for 3.5 h of incubation, whereas at 24 h no effect of vegetation cycle was observed for the C2:C3 ratio.

3.6 Ammonia nitrogen (NH_3-N)

Stems and leaves

At 3.5 h of incubation, by the presence of CT, the NH_3-N content in the incubation medium differed significantly between stems and leaves only at the first vegetation cycle (end of flowering) ($P < 0.001$) and the values were higher for stems than for leaves. However, at 24 h of incubation there were no significant differences between leaves and stems at either vegetation cycle. For both incubation times, added PEG increased NH_3-N ($P < 0.001$) for leaves and stems at both vegetation cycles, more markedly at the second one($P < 0.001$) (Table 3 and Table 4). During both incubation periods the differences between with and without PEG addition were greater for leaves than for stems at both vegetation cycles.

Whole plant

Addition of PEG resulted in higher NH_3-N content ($P < 0.001$) at both 3.5 h and 24 h of incubation (Table 5 and Table 6). There was no significant difference between the mean values for the two vegetation cycles at 3.5 h of incubation. However, at 24 h of

incubation the mean values obtained at the second vegetation cycle (start of flowering) were higher than at the end of flowering (0.653 *vs*. 0.491) ($P < 0.001$). At 24 h of incubation the differences between the two vegetation cycles were greater with added PEG (0.530 *vs.* 0.823) than without PEG (0.453 *vs.* 0.483).

4. Discussion

We demonstrate the *in vitro* effect of CT on N digestion and organic matter digestibility, and on gas production, especially of methane (CH_4). We focus on the effects of CT on rumen fermentation characteristics for different parts of sainfoin at two vegetation cycles. The relation between content, structure and distribution of CT among sainfoin leaves, stems and whole plant according to vegetation cycle were thoroughly discussed in a previous paper (Theodoridou et al., 2010).

Here the biological activity and prodelphinidin (PD) content were higher ($P < 0.001$) for leaves than for stems, whereas the *cis* value was lower ($P < 0.01$). A marked variation in the mDP values was obtained, comparing the Perly variety used in this study with other sainfoin varieties (data not shown).

4.1 Methane and gas production

There was a considerable increase in gas production when stems, leaves or whole plant were incubated in the presence of inactivated CT. This result is consistent with those of Karabulut et al. (2006) obtained with *L. corniculatus* harvested at the flowering stage, those of Seresinhe and Iben (2003) with *Gliricidia sepium* and *Calliandra calothyrsus* and those of Getachew et al. (2001) with tannin-containing browses (*Acacia albida, Acacia cyanophylla* and *Calliandra calothyrsus*).

The increase in gas production in the presence of PEG is due to an increase in the nutrients available to rumen microorganisms, especially available nitrogen (Karabulut et al., 2006). However, according tofor Getachew et al. (1998), addition of PEG to tannin-containing browse and herbaceous legumes increased *in vitro* gas production, but decreased microbial nitrogen and the efficiency of microbial protein synthesis. The different observations in previous studies may be attributed to differences in biological activity and CT content among different plants. Rumen digestion of feed components by the microbiota (bacteria, protozoa, fungi), under anaerobic conditions, results in the production of gases (CO_2 and CH_4) eliminated through eructation (Martin et al., 2010). CH_4-producing archaea, known as methanogens, are distinct groups of organisms, which form a normal component of the rumen microbial ecosystem (Tavendale et al., 2005). In our study, at 3.5 h and 24 h of incubation, CH_4 production was increased by the presence of PEG for leaves and stems. Our results were in line with those of Woodward *et al*. (2004), who found that CH_4 production was reduced by 13% in *L. corniculatus* by the presence of CT. Also, Tavendale et al. (2005), in *L. pedunculatus,* observed that CT decreased CH_4 production, and Waghorn et al. (2002) showed a 31% reduction from sheep-fed *L. peduncularus* compared with *M. sativa* (tannin-free legume). Bhatta et al. (2009) found that CH_4 production *in vitro* from six plant sources of condensed tannins (CT) and hydrolysable tannins (HT) was decreased in the presence of CT.

In contrast with our observations and the other studies cited above, Beauchemin et al. (2007) found that feeding up to 2% of the dietary DM as quebracho tannin extract failed to reduce enteric methane emissions from growing cattle. The authors conclude that CTs extracted from different plants range widely in their capacity to bind

carbohydrates and proteins. Thus quebracho tannin extract may be a less effective CH_4 suppressant than other CT sources. The comparison of leaves and stems, and of two vegetation cycles in our study clearly shows that the effect of CT on gas production depends on concentration and physical and chemical structure. The higher gas and CH_4 production observed for stems was readily explained by their lower biological activity ($P < 0.001$), as well as a lower PD value ($P < 0.001$) than leaves. It is known that the lower PD content, in relation with a lower biological activity, decreases the ability of CT to complex with proteins (Jones *et al*., 1976; Aerts et al., 1999). The lower CH_4 production in sainfoin could be due to the anti-methanogenic activity of CT and their interaction with rumen bacteria (McAllister et al. 1994; Puchala et al., 2005; Martin et al., 2010). Two modes of action of CT on methanogenesis have been proposed: (i) a direct effect on ruminal methanogenic bacteria and archaea, and (ii) an indirect effect on fibre digestion, decreasing production of hydrogen, a substrate for these bacteria (Tavendale et al., 2005). Hydrogen elimination can also occur during the synthesis of volatile fatty acids. Acetate and butyrate promote CH_4 production, while propionate formation can be considered as a competitive pathway for hydrogen use in the rumen (Moss et al., 2000).

4.2 In vitro true OM digestibility (IVTOMD) and nitrogen disappearence (ND)

In our study, IVTOMD of stems, leaves and the whole plant was not increased when CT were inactivated by added PEG at either 3.5 h or 24 h of incubation. This is consistent with measurements made *in vivo* with the same plants (Theodoridou et al, 2010) and indicates that CT in sainfoin at these concentrations have no negative effect on the digestibility or energy value of the plant.

It is known that tropical legume plants contain large quantities of CT (Ben Salem, 1998), which are not expected to be nutritively beneficial (Ben Salem, 1998; Barry and McNabb, 1999). Barry and McNabb (1999) advised that dietary concentrations lower than 50 gCT/kg DM would be beneficial for animal health and nutrition. However, these recommendations require caution: reported CT contents depend on the analytical method used, and they originate mainly from feeding trials with *Lotus* species that may not be applicable extendable to other feeds (Waghorn and McNabb, 2003; Mueller-Harvey, 2006).

Also at 24 h of incubation, IVTOMD decreased with added PEG. The same results were obtained *in vitro* by Tiemann et al. (2008) on tropical tanniniferous legumes. Other published studies found an increase in IVTOMD with added PEG (Sandoval et al. 2002 on tropical legumes; Karabulut et al. 2006 on *L. corniculatus*; Madibela et al. 2006 on *Acacia*). The decreased IVTOMD observed with added PEG may be due to complex insoluble artefacts formed with other component of the feed, such as fibre, remaining in the residue, resulting in an underestimation of IVTOMD (Palmer and Jones 2000; Makkar, 2000)

At both incubation times N disappearance (ND) increased with added PEG, in line with the results of Tiemann et al. (2008) on tanniniferous tropical plants and of Getachew et al. (2000) on tannin-rich browse and herbaceous legumes. At 3.5 h of incubation N disappearance was lower for leaves than for stems at two vegetation cycles, whereas at 24 h of incubation, there were no differences between leaves and stems. These results were unexpected, as leaves had higher N content than stems. This finding may be partly explained by the greater indigestible nitrogen concentrations in leaves than in stems.

Leaves, because of their greater amounts of primary cell walls, would be expected to have greater concentrations of cell wall N than stems, which have more secondary cell walls (Sanderson and Wedin, 1989).

In addition, the lower N disappearance of leaves may be due to their higher CT biological activity and PD value. More CT binding protein in leaves results in a lower N disappearance in leaves than in stems. In a previous *in vivo* study on the same forages, Theodoridou et al., 2010 observed that N disappearance of leaves from nylon bags in the rumen was lower than that of stems.

PF (TDOM/gas produced) was defined by Blummel et al. (1997) as an indicator of the partitioning of TDOM between microbial biomass and fermentation gases during fermentation. In our study we found that the PF of leaves, stems and whole plant was increased ($P < 0.01$) at both incubation periods in the presence of CT. This was in accordance with a decrease in total gas production ($P < 0.001$), while the IVTOMD was not decreased by CT. The higher PF value due to CT could indicate an increase in the microbial protein synthesis in the medium.

4.3 NH3-N production

The main problem in using forage legumes is that their proteins are rapidly degraded in the rumen, leading to N losses via urinary urea excretion (Min et al., 2003).

In our study, sainfoin CT decreased NH_3-N production of leaves, stems and whole plant in the medium at both 3.5 h and 24 h of incubation as shown previously in an *in vitro* continuous culture system (Dahlberg et al., 1988) and on sainfoin incubated alone by Niderkorn et al., 2010). The effect of CT on NH_3-N production was consistent with the results obtained in our *in vivo* study (Theodoridou et al., 2010), whereas sainfoin CT

decreased rumen fluid NH_3-N content. The results in the present study were not surprising, since CT decrease ruminal protein degradation by binding dietary proteins in the rumen and inhibit excessive protein degradation by reducing proteolytic enzyme activity (Jones and Mangan, 1977; Waghorn et al., 1998; Aerts et al., 1999; Turner and Neel, 2003).

The difference between NH_3-N productions measured with and without added PEG was markedly higher at the second vegetation cycle (start of flowering) for all parts of the plants at both 3.5 h and 24 h of incubation. This may be due to the higher N content in a less mature plant, and also due to a higher CT content, biological activity, and lower mean degree of polymerisation.

4.4 VFA and *C2:C3 ratio*

Gas production is basically the result of fermentation of carbohydrates to acetate, propionate and butyrate.

VFA production was increased with added PEG for stems and leaves at both short and long fermentation times. These results are consistent with those of Mbugua et al. (2008) in tropical herbaceous and tree forage legumes and Salawu et al., (1997) in *Calliandra calothyrsus.* According to Getachew et al. (2001) addition of PEG to tannin-containing legumes increased *in vitro* gas VFA and short chain fatty acid production, but decreased microbial nitrogen and efficiency of microbial protein synthesis. The differences obtained with and without PEG were higher for leaves than for stems, in accordance with their CT characteristics. The increased C2:C3 ratio in the presence of CT was in relation with the slower rate of digestion (Waghorn 2008).

By contrast, for the whole plant, VFA production and C2:C3 ratio was not significantly affected by CT after long incubation (24 h). This may be due to the adaptation of the rumen ecosystem to CT (Mlambo et al., 2007). Rumen microbes have the ability to adapt to dietary phenolics and CT (Sarisalo et al., 1999; McSweeny et al., 2001) by different mechanisms, such as changes in microbial population, synthesis of tannin-degrading enzymes (Pell et al., 2000, Murdiati et al., 1992) or excretions of tannin-binding polysaccaharides (Brooker et al., 2000).

Conclusion

This *in vitro* study confirms the role of CT in sainfoin in N digestion and gas production from the rumen ecosystem. Comparison between leaves and stems, which would be difficult to perform *in vivo*, shows that the effects of CT are greater in leaves, in accordance with their higher biological activity and PD value. Incubation of leaves had a greater effect on *in vitro* fermentation than that of stems. This could be due to the different CT characteristics between these two parts of the plant. Thus differences in CT activity between leaves and stems contribute to the differences between vegetation cycles at the whole plant level.

The utilisation of tanniniferous plants such as sainfoin curbs production of CH_4, one of the three main greenhouse gases, without decreasing *in vitro* true organic matter digestibility (IVTOMD). The decrease in N disappearance measured *in vitro* was promising for reducing the degradation of proteins in the rumen and increasing the outflow reaching the intestine.

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Table 1 Chemical composition and pepsin-cellulase digestibility (Dcell) (g/kg DM) of sainfoin studied as fresh forage at the first vegetation cycle (end of flowering) and the second vegetation cycle (start of flowering).

	First vegetation cycle			Second vegetation cycle					Statistical significance	
Phenological stage	End of flowering			Start of flowering			*n*	SEM	VC	PP
Proportion of leaves in the whole plant	0.360			0.470						
	Leaves (g/kg DM)	Stems (g/kg DM)	Whole plant (g/kg DM)	Leaves (g/kg DM)	Stems (g/kg DM)	Whole plant (g/kg DM)				
N	32.7	16.1	22.9	37.1	22.3	29.9	3	0.11	***	***
aNDF	255	526	415	253	412	355	3	5.77	***	***
ADF	215	408	314	183	322	279	3	3.8	***	***
Lignin(sa)	ND	ND	67.4	ND	ND	77.7	3	2.48	NS	***
Dcell	ND	ND	557	ND	ND	610	3	4.3	NS	ND

SEM: standard error of mean; NS, not significant ($P > 0.05$).
Probability of significant effects due to vegetation cycle (VC) and the part of the plant (PP) in the analysis for fresh forages (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Table 2
Characterisation of condensed tannins (CT) of sainfoin studied as fresh forage at the first vegetation cycle (end of flowering) and the second vegetation cycle (start of flowering).

	First vegetation cycle			Second vegetation cycle					Statistical significance	
Phenological stage	End of flowering			Start of flowering			*n*	SEM	VC	PP
	Leaves	Stems	Whole plant	Leaves	Stems	Whole plant				
CT biological activity (g tannic acid equivalent/100g)(DM)	1.97	1.00	1.02	3.40	1.23	1.58	3	0.142	***	***
CT content (g/kg DM)	5.0	4.3	6.2	9.8	9.0	13.6	3	0.20	NS	NS
CT mean degree of polymerisation (mDP)	37.4	24.6	31.0	11.8	8.2	11.0	3	4.44	**	NS
CT prodelphinidin (PD)	80.4	54.1	69.2	76.4	55.4	70.5	3	0.45	NS	***
CT cis	64.6	84.4	82.9	72.8	83.1	81.7	3	2.40	NS	**

SEM: standard error of mean; NS, not significant ($P > 0.05$).
Probability of significant effects due to vegetation cycle (VC) and the part of the plant (PP) in the analysis for fresh forages (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Table 3
In vitro rumen fermentation characteristics obtained from 3.5 h fermentation of stems and leaves of sainfoin at the first vegetation cycle (end of flowering) and the second vegetation cycle (start of flowering).

	Stems				Leaves				Statistical significance					
	First vegetation cycle		Second vegetation cycle		First vegetation cycle		Second vegetation cycle		SEM	PP	T	VC	T*VC	T*PP
Phenological stage	End of flowering		Start of flowering		End of flowering		Start of flowering							
PEG treatment	With	without	with	without	with	without	With	without						
pH	6.78	6.76	6.65	6.69	6.68	6.73	6.64	6.76	0.028	**	**	***	*	*
Gas production mmol/batch														
Gas-3.5 h	1.250	1.188	1.512	1.311	1.436	1.072	1.603	1.033	0.0655	*	***	***	**	***
CH_4	0.293	0.250	0.250	0.210	0.237	0.173	0.320	0.140	0.0159	*	***	ns	*	**
CO_2:CH_4, mol/mol	3.633	4.190	5.093	5.597	5.217	5.960	4.740	6.783	0.3482	***	**	**	ns	ns
IVTOMD %	44.857	45.103	57.220	58.003	64.567	64.947	64.953	65.633	1.3704	***	ns	***	ns	ns
N disappearance %	76.963	76.763	85.093	82.767	80.407	75.567	81.623	76.840	2.6774	**	**	***	ns	ns
PF, mg TDOM/mL gas	6.733	7.267	6.933	8.523	8.107	10.703	7.517	11.777	0.3290	***	***	*	**	**
End products, mmol/batch														
NH_3-N	0.127	0.110	0.187	0.077	0.263	0.070	0.330	0.070	0.0085	***	***	**	***	***
Total VFA	1.583	1.553	1.910	1.783	1.907	1.547	1.997	1.480	0.0860	**	***	***	ns	***
C2	1.123	1.107	1.353	1.290	1.350	1.123	1.437	1.103	0.0576	**	***	***	ns	***
C3	0.392	0.383	0.473	0.430	0.480	0.367	0.480	0.327	0.0241	***	***	*	*	***
C4	0.070	0.0633	0.080	0.067	0.080	0.053	0.087	0.047	0.00635	ns	***	ns	ns	**
ratio CH_4:TDOM	1.452	1.031	0.887	0.707	0.853	0.623	0.961	0.454	0.0918	**	***	*	ns	ns
ratio C2:C3	2.847	2.880	2.863	3.0300	2.830	3.0467	3.017	3.377	0.0606	***	***	***	*	*

SEM: standard error of mean; NS, not significant ($P > 0.05$).
Probability of significant effects due to vegetation cycle (VC), treatment (T) and part of the plant (PP) in the analysis for fresh forages (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Table 4
In vitro rumen fermentation characteristics obtained from the 24 h fermentation of stems and leaves of sainfoin at the first vegetation cycle (end of flowering) and the second vegetation cycle (start of flowering).

	Stems				Leaves				Statistical significance					
	First vegetation cycle		Second vegetation cycle		First vegetation cycle		Second vegetation cycle		SEM	PP	T	VC	T*VC	T*PP
Phenological stage	End of Flowering		Start of flowering		End of Flowering		Start of flowering							
PEG treatment	with	without	with	without	with	Without	with	without						
pH	6.46	6.44	6.38	6.35	6.44	6.42	6.49	6.46	0.023	***	*	*	ns	ns
Gas production mmol/batch														
gas-24 h	3.260	3.146	3.513	3.281	3.420	3.031	3.492	3.008	0.0581	*	***	**	ns	**
CH_4	0.837	0.862	0.857	0.767	0.793	0.692	0.894	0.497	0.0440	*	**	ns	*	*
CO_2:CH_4, mol/mol	2.440	2.495	2.112	1.842	1.925	1.632	2.188	1.294	0.1154	**	**	*	*	ns
IVTOMD %	62.623	62.910	73.333	75.107	78.347	80.717	77.257	82.797	1.6479	***	***	***	*	*
N disappearance %	81.227	79.283	87.347	83.693	84.160	78.903	86.030	83.280	2.8826	ns	**	**	ns	ns
PF, mg TDOM/mL gas	3.800	3.937	4.173	4.533	4.373	5.040	4.283	5.300	0.1336	***	***	***	*	**
End products, mmol/batch														
NH_3-N	0.463	0.460	0.617	0.470	0.863	0.450	1.120	0.530	0.0809	***	***	***	***	***
Total VFA	3.107	3.100	3.390	3.443	3.567	3.173	3.550	3.163	0.1118	***	***	***	ns	***
C2	2.143	2.150	2.343	2.3967	2.4667	2.230	2.480	2.293	0.0702	***	**	***	ns	***
C3	0.703	0.693	0.767	0.760	0.813	0.697	0.777	0.637	0.0170	***	***	ns	ns	***
C4	0.260	0.260	0.280	0.293	0.2867	0.243	0.293	0.230	0.0301	*	***	ns	ns	***
ratio CH4:TDOM	2.440	2.495	2.112	1.8412	1.925	1.632	2.188	1.294	0.1154	**	**	*	*	ns
ratio C2:C3	3.053	3.103	3.070	3.160	3.030	3.203	3.193	3.607	0.0687	***	***	**	ns	*

SEM: standard error of mean; NS, not significant ($P > 0.05$).
Probability of significant effects due to vegetation cycle (VC), treatment (T) and part of the plant (PP) in the analysis for fresh forages (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Table 5
In vitro rumen fermentation characteristics obtained from 3.5 h fermentation of the whole plant of sainfoin at the first vegetation cycle (end of flowering) and the second vegetation cycle (start of flowering).

	Whole plant				Statistical significance			
	First vegetation cycle		Second vegetation cycle					
Phenological stage	End of flowering		Start of Flowering		SEM	T	VC	T*VC
PEG treatment	with	without	with	without				
pH	6.75	6.76	6.68	6.71	0.032	ns	**	ns
Gas production mmol/batch								
gas-3.5 h	1.222	1.157	1.560	1.234	0.0542	***	***	****
CH_4	0.240	0.197	0.257	0.210	0.0208	ns	ns	ns
CO_2:CH_4, mol/mol	4.583	4.850	5.250	5.350	0.3670	ns	ns	ns
IVTOMD %	50.073	50.463	60.790	61.0467	1.102	ns	***	ns
N disappearance, %	79.550	78.637	86.063	83.290	2.8450	ns	***	ns
PF, mg TDOM/mL gas	7.623	7.997	7.610	9.287	0.1866	**	*	*
End products, mmol/batch								
NH_3-N	0.170	0.110	0.243	0.060	0.0067	***	ns	***
Total VFA	1.687	1.533	1.987	1.623	0.0722	***	***	ns
C2	1.190	1.087	1.423	1.183	0.0468	***	***	**
C3	0.420	0.383	0.477	0.380	0.0191	***	*	*
C4	0.077	0.067	0.0833	0.060	0.0041	***	ns	*
ratio CH_4:TDOM	1.021	0.777	0.785	0.625	0.0839	**	*	ns
ratio C2:C3	2.857	2.830	2.977	3.130	0.0439	ns	***	*

SEM: standard error of mean; NS, not significant ($P > 0.05$).
Probability of significant effects due to vegetation cycle (VC), treatment (T) in the analysis for fresh forages (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Table 6
In vitro rumen fermentation characteristics obtained from 24 h fermentation of the whole plant of sainfoin at the first vegetation cycle (end of flowering) and the second vegetation cycle (start of flowering).

	Whole plant				Statistical significance			
	First vegetation cycle		Second vegetation cycle					
Phenological stage	End of flowering		Start of flowering		SEM	T	VC	T*VC
PEG treatment	With	without	with	without				
pH	6.45	6.45	6.41	6.38	0.023	ns	**	ns
Gas production mmol/batch								
gas-24 h	3.272	3.075	3.560	3.221	0.0620	***	***	*
CH_4	0.783	0.7077	0.870	0.800	0.0469	ns	*	ns
CO_2:CH_4, mol/mol	3.067	3.148	3.107	3.267	0.1643	ns	ns	ns
IVTOMD %	67.787	68.047	74.730	76.650	1.1409	ns	***	ns
N disappearance, %	84.527	82.267	88.233	85.540	3.0917	*	*	ns
PF, mg TDOM/mL gas	4.080	4.323	4.1367	4.690	0.1271	***	**	*
End products, mmol/batch								
NH_3-N	0.530	0.453	0.823	0.483	0.0780	***	***	***
Total VFA	3.217	3.057	3.260	2.750	0.2570	ns	ns	ns
C2	2.220	2.143	2.250	1.923	0.1854	ns	ns	ns
C3	0.727	0.673	0.737	0.600	0.05701	ns	ns	ns
C4	0.273	0.250	0.277	0.230	0.0272	ns	ns	ns
ratio CH4:TDOM	2.137	1.924	2.141	1.848	0.0998	*	ns	ns
ratio C2:C3	3.070	3.187	3.077	3.553	0.1683	ns	ns	ns

SEM: standard error of mean; NS, not significant ($P > 0.05$).
Probability of significant effects due to vegetation cycle (VC), treatment (T) in the analysis for fresh forages (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

PART 3

How the preservation method modified the content of condensed tannins and their effects on the sainfoin digestion and its nutritive value

Submitted to *Animal*

Effects of condensed tannins in wrapped silage bales of sainfoin (*Onobrychis viciifolia*) on *in vivo* and *in situ* digestion in sheep

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Abstract

The objective of the current study was to characterize the condensed tannins (CT) in wrapped silage bales of sainfoin (*Onobrychis viciifolia*) and examine their potential action on *in vivo* and *in situ* digestive characteristics in sheep. Silage was made from sainfoin at two phenological stages. The first phenological stage at which silage was made was from first cycle vegetation growth at the end of flowering and the second stage silage was made from regrowth, five weeks after the first cut, and prior to flowering. The silages made from the two phenological stages were fed to 12 rumen-fistulated sheep in a crossover design. Six received polyethylene glycol (PEG) to bind with and remove the effects of CT, while the other six were dosed with water. Organic matter digestibility (OMD), total tract N digestibility and nitrogen (N) balance were measured over 6 days. Kinetic studies were performed on total nitrogen (tN), ammonia nitrogen (NH_3-N) and volatile fatty acids (VFA) in rumen fluid before and 1.5 h, 3 h and 6 h after feeding. The kinetics of degradation of dry matter (DM) and nitrogen (N) from Dacron bags suspended in the rumen were also determined. Biological activity of CT (protein binding capacity) and CT content were greater for the silage made from sainfoin at the early of flowering stage. Total-tract N digestibility was increased by the addition of PEG (P<0.001) for the sainfoin silage prior to

flowering (P<0.001). Condensed tannins increased N excretion in urine (P<0.05) and decreased faecal N excretion (P<0.001), but had no effect on body N retention, which is beneficial for the animal. Ruminal nitrogen degradability (DegN) was lower in the presence of active CT (P<0.001) at both phenological stages, but soluble N (P=0.2060) and NH_3-N (P=0.5225) contents in rumen fluid remained unchanged. The results of this experiment indicate that the CT in the sainfoin keep their ability to affect the nutritive value of preserved forage legumes.

Keywords: Feed value, *in vivo* measurements, tannins, polyethylene glycol, sainfoin silage.

Introduction

Condensed tannins (CT) are polyphenols that are capable of binding to protein which slows the degradation of dietary protein in the rumen and during ensiling (Min *et al.,* 2003, Waghorn, 2008). Preserving legumes as silage is a good way to provide an on-farm source of home-grown energy and protein, and offers advantages over traditional haymaking since it is less weather-dependent and allows for high quality forage preservation (Cavallarin *et al.*, 2004).

Numerous papers have focused on the effect of CT on the nutritive value of fresh forages, but few reports give data on preserved tanniniferous forages and for that reason there is a need to investigate the effects of the ensiling process on the nutritive value of CT-containing forages and on the activity of CT.

The objective of this study was to investigate the effect of ensiling sainfoin at two phenological stages on the *in vivo* digestibility and nitrogen balance and *in situ* rumen degradability in sheep. The effect of CT on ensiling process was assessed by

addition of polyethylene glycol (PEG), which forms complexes with CT and inactivates them, throughout the entire digestive tract (Barry *et al*., 2001).

Material and methods

The study was conducted indoors at the INRA-UR1213 experimental farm in France (45°42'N, 03°30'E). The animals were handled by specialized personnel who applied animal care and welfare in accordance with European Union Directive No. 609/1986, under agreement No. A63 565.

Forage management and silage making

Sainfoin (*Onobrychis viciifolia*) cv. Perly was grown in Clermont-Ferrand (France) on the INRA's Crouël site at an altitude of 320 m on a fertile basic loamy soil (37.4% sand, 45.8% loam, 16.8% clay, 3.7% organic matter, pH 8.2). No mineral fertilizer was applied. The experiments were conducted during 2008 with wrapped silage bales of sainfoin made from fresh forage harvested at the end of flowering (50% of stems showed open flowers on the top half) during the first vegetation cycle (Silage 1) and at the early flowering (50% of stems showed open flowers on the lowest half of the flower stem) during the second vegetation cycle (Silage 2). Sainfoin yield was 8.5 $\pm$ 1.1 t of dry matter (DM) /ha $\pm$ 1.1 and 3.3 t DM/ha, respectively, for each sainfoin cut.

The fresh forages were cut with a mower conditioner ('VICON' type 3001 HPC, Merseyside, UK). The forages were wilted for one day after cutting, then made into wrapped silage bales using a fixed chamber baler (Welger, RP 200, type 1721, Kverneland, Blanchot, avenue de l'Europe 02402 Château- Thierry Cedex FRANCE) and stored until use.

Animals and experimental design

Twelve-cannulated castrated male Texel sheep (12 months old, 60±3 kg liveweight) were used for digestibility and N balance measurements (Demarquilly et al., 1995), ruminal fluid sampling, and rumen degradability measurements on wrapped silage bales of sainfoin (Michalet-Doreau et al., 1987, modified by Dulphy et al., 1999). The sheep were drenched with anthelmintic (Ivomec, Merial, France) 30 days before start the experiment to eliminate internal and external parasites.

All the sheep were offered 60 g DM/kg $BW^{0.75}$ of wrapped silage bales of sainfoin daily in two meals per day at 8 a.m. and 4 p.m. One group of six sheep was dosed daily with 60 g of PEG 4000 (2 x 30 g in 200 ml water) delivered through the rumen cannula before each meal, while the other group was dosed with 2 x 200 ml of water (without PEG) (Figure 1).

During the experimental periods, the animals were housed in individual crates for *in vivo* and *in situ* measurements. They were given *ad libitum* access to water and a salt block.

Wrapped silage bales of sainfoin were tested in an incomplete cross-over design experiment in two periods. Each experimental period included a two-week adaptation phase and two weeks for measurements: the first six days to measure digestibility, N balance and kinetics of ruminal fluid, and the second week to measure *in situ* degradation kinetics. In the first experimental period, 3 sheep from each group (with or without PEG) were randomly assigned to receive wrapped silage bales of sainfoin harvested at the first vegetation cycle (Silage 1) while the other 3 sheep received wrapped silage bales of sainfoin harvested at the second vegetation cycle (Silage 2). In the second experimental period, the sheep receiving silage 1 were switched over to silage 2, and vice versa.

Forage and condensed tannins analyses

Sample collection

Four representative samples obtained from silage 1 and from silage 2 were used for the analyses of forage chemical composition, ensiling characteristics and of condensed tannins.

All the analyses for CT content and biological activity were performed on freeze-dried plant material. Analyses of forage chemical composition were performed on dried in a forced air oven at 60° C for 72 h and grounded (1 mm sieve) samples and analyses of the silage fermentative characteristics on silage juice.

Laboratory analyses

Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) of the offered wrapped silage bales of sainfoin were analyzed in filter bags according to Van Soest and Wine (1967) modified by Pagan *et al.* (2009) and nitrogen content was analysed using Kjeldahl method. Detergent insoluble nitrogen (NDIN) and acid detergent insoluble nitrogen (ADIN) were determined on offered wrapped silage bales of sainfoin. Pepsin-cellulase digestibility was determined according to Aufrère and Michalet-Doreau (1983). Rumen VFA content was analyzed by gas chromatography (CHROMPACK CP 9002, France) according to Jouany (1982).

Analyses were run on the fermentative characteristics of the wrapped silage bales of sainfoin (pH, NH3-N, soluble N, VFA, lactic acid).

Condensed tannins contents (extractable and non-extractable tannins) were measured at two vegetation cycles by HCl-butanol (Reed, 1986). The biological activity of CT in terms of protein-binding capacity was determined using the radial diffusion assay (RDA). This assay measures precipitation of bovine serum albumin

(BSA) by tannins, with the area of the precipitation ring being proportional to the amount of extracted CT. CT extraction from freeze-dried samples was performed using a 70% acetone solution (Makkar *et al.*, 2000).

In vivo digestibility and N balance

Sample collection

For digestibility and N balance measurements, during the experimental period, both sheep groups were kept in metabolic crates where daily, total urine was funnelled with a spout into the collection flask and total faeces were collected in a bin, located behind the sheep. Loss of ammonia from urine was prevented by daily addition of 50 ml of 30% (w/v) sulphuric acid to the collection flask. Daily aliquots of faeces and urine were pooled per sheep for the 6 days of measurements. During this week, the sainfoin wrapped silage bales offered were sampled daily, weighed, and then fed to the sheep.

Laboratory analyses

Every day, feed samples and refusals were dried in a forced-air oven at 60°C for 72 h for DM determination. All samples were ground through a 1-mm sieve before analysis of N content using Kjehldahl method. Pooled samples of urine were stored at -20°C for later analysis of total N content using Kjehldahl method.

Ruminal fluid sampling

Sample collection

Over 4 consecutive days, rumen fluid was sampled from three sheep each day, i.e. the 6 sheep with PEG over the first two days and the 6 sheep without PEG over the

following two days (Figure 1). Sampling was performed before the morning meal (T0) and at 1.5 h, 3 h and 6h after feeding.

Laboratory analyses

At each timepoint, 250 ml of rumen fluid was sampled, measured for pH using a pH meter (Mettler SevenEasy S20, France) and filtered through layers of muslin. After filtration of rumen fluid the solid particles were re-introduced in the rumen. The rumen fluid was then centrifuged for 5 min at 3000*g* at 4°C to remove large dietary particles. Samples for the analysis of volatile fatty acids (VFA) were obtained by taking 0.8 ml from the supernatant mixed with 0.5 ml of crotonic acid and then centrifuged at 14,000 *g* for 10 min at 4°C. The supernatant from the first centrifugation of rumen fluid was centrifuged again at 4°C and 27,000*g* for 20 min to remove small dietary particles and bacteria. In the supernatant the tN was analysed using Kjehldahl method and ammonia nitrogen (NH_3-N) was determined according to Weatherburn (1967).

In situ degradation in the rumen

Sample collection

Dry matter and and nitrogen degradation in the rumen were measured via the nylon bag procedure using Dacron bags (pore size 53 ± 15 µm, Ankom Co., NY, USA) with an internal surface area of 5 x 11 cm and closed by two stitches. For each phenological stage, after opening up the sainfoin wrapped silage bales, representative samples from the bale were collected, cut into 1 cm lengths to mimic sheep mastication, and placed in the Dacron bags. The bags were filled with a forage quantity equivalent to 2.5 g DM, heat-sealed, then quickly frozen in liquid nitrogen and stored at -20°C before use.

In situ degradation was measured in the rumen of the 12 sheep (six with and six without PEG) after incubation times of 2, 4, 8, 16, 24 and 48 h, with 2 bags for 2, 4 and 8h and 3 bags for 16, 24 and 48 h (Figure 1). Samples of a common grass hay used as a standard were incubated in duplicate for 8 h in the rumen of each animal to detect any changes in level of rumen degradation activity during the experiment.

Laboratory analyses

After removal from the rumen, the bags were stored at -20°C until washing. They were then thawed and washed in a washing machine (3 x 10 min) under a cold-water cycle until the rinse water was clear. The bags were then beaten for 7 min in a "stomacher" (Stomacher 80 SEWARD, France) to reduce microbial contamination of the undegraded sample fraction, re-washed (2 x 10 min), and freeze-dried. A correction to take into account adherent bacteria not detached by this treatment was performed according to Michalet-Doreau and Ould-Bah (1989). The soluble N fraction was determined by soaking the bags containing the samples in warm water (40°C) for 1.5 h, and was considered as the proportion of forage N that had disappeared from the bags at T0 h. All the bags were freeze-dried before analysis. The residues obtained from *in situ* incubation were analyzed for N by the Dumas method (Buckee 1994).

Calculations

The *in situ* rumen DM and N disappearance curves of wrapped silage bales of sainfoin were fitted to the model of Ørskov and McDonald (1979) using a non-linear regression procedure (SAS, 2000). The proportion of N degraded at time *t* was calculated using the equation: N degraded = $a + b\ (1- e^{-ct})$, where *a* is the rapidly degraded fraction, *b* is the slowly degraded fraction, and *c* is the degradation rate of

the slowly degradable fraction, while (100 − *a* – *b*) was considered the undegradable fraction. The effective degradability of N (DegN) was calculated according to the equation: DegN = *a* + *bc* / (*c* + k_p), where fractional passage rate k_p is assumed to be equal to 0.06/h.

The same model was used to calculate the effective degradability of DM (DegDM).

Statistical analysis

Data were analyzed using the Mixed Procedure of the SAS software package (2000). Data related to forage chemical composition were analyzed according to a model including phenological stage. Data from *in vivo* and *in situ* measurements were submitted to ANOVA to test phenological stage and treatment (with or without PEG) as fixed effects, and sheep nested within treatment and experimental period as random effects. The model included the interaction between treatment and phenological stage. Rumen fluid data was submitted to repeated-measures ANOVA, with Phs and T as fixed effects, and sheep nested within treatment and experimental period as random effects. Time was considered as a repeated variable. The model included also the interactions between time, treatment and phenological stage.

Results

Chemical composition and characteristics of condensed tannins

The chemical composition of the wrapped silage bales of sainfoin is presented in Table 1. The dry matter content (g/kg DM) was greater (P<0.05) in the wrapped silage bales made from sainfoin at the early flowering (398) compared to that made at the end of flowering stage (375). Nitrogen content of the wrapped silage bales of sainfoin at the end of flowering was 22.4 g/kg DM, and was lower (P<0.01) than at

the early flowering (27.8). The fibre content (NDF and ADF) of sainfoin as wrapped silage bales increased markedly with increasing plant maturity, reaching higher values (P<0.001) at the end than the early flowering. Detergent insoluble nitrogen (P<0.001) and acid detergent insoluble nitrogen content (P<0.01) in wrapped silage bales of sainfoin were lower at the early than the end of flowering. The increasing cell-wall content led to a decrease in pepsin-cellulase digestibility (P<0.001).

Protein binding capacity of CT and their content (extractable and non-extractable tannins) in wrapped silage bales of sainfoin were higher at the early flowering (1.10 eq tannic acid and 33.9 g/kg DM) compared to the end of flowering (0.78 eq tannic acid and 17.6 g/kg DM) (Table 1).

Ensiling characteristics in wrapped silages

The fermentation parameters of the wrapped silage bales of sainfoin are reported in Table 2. The wrapped silage bales of sainfoin at the end of flowering had a higher butyric acid content (P<0.001), propionic acid content (P<0.01) and higher pH (P<0.01). The pH increased about 0.3 points between the early flowering (4.61) and the end of flowering (4.91). Lactic acid was the major acid found, at 22.76 and 28.87 g/kg DM at end of flowering and early flowering, respectively. Acetic acid production (g/kg DM) tended to be higher at the end of flowering (6.83) compared to the early flowering (5.68). The content of ethanol, a predominant fermentation product, was not significantly different between the two phenological stages. Methanol content was lower (P<0.001) at the end of flowering (0.38) compared to the early flowering (1.16). Soluble-N and NH3-N levels expressed in % N were lower (P<0.001) at early flowering than end of flowering (36.09 and 3.82 vs 45.81 and 7.87, respectively).

In vivo digestibility and nitrogen balance

Organic matter digestibility (OMD) was higher (P<0.001) at the early flowering than the end of flowering, but PEG addition only affected OMD at the end of flowering (P<0.01).

Without the addition of PEG, N digestibility was higher (P<0.001) at the early flowering compared to the end of flowering (Table 3). Total-tract N digestibility increased markedly with the addition of PEG (P<0.001) at both phenological stages. However, the amount of N retained was not significantly affected by the addition of PEG (P>0.05), and was significantly higher at the early flowering. Adding PEG increased N excretion in urine (P<0.05) and decreased fecal N excretion (P<0.001).

In situ dry matter and nitrogen degradability in the rumen

There was no difference in DegDM between the two phenological stages (Table 4). By contrast, there was an effect of phenological stage on the DegN of sainfoin, which was higher at end of flowering than at early flowering (P<0.001). Adding PEG significantly increased the DegDM (P<0.01) and DegN (P<0.001) of wrapped silage bales of sainfoin. For both DM and N, the soluble fraction ‘a’ and the potentially degradable fraction ‘b’ were unaffected by the addition of PEG, in contrast to the degradation rate ‘c’ which was increased (P<0.001).

Rumen fluid composition

Total nitrogen and ammonia-N contents in rumen fluid peaked at 1.5 h after feeding and decreased until 6 h after feeding (Figure 2, Figure 3), and were higher (P<0.001) at the early flowering than at the end of flowering. Adding PEG did not significantly increase tN and NH3-N contents (Table 4). Whatever the treatment, VFA contents

were higher (P<0.001) at the early flowering compared to the end of flowering. Adding PEG had no significantly effect on VFA content. Acetate-to-propionate ratio was higher (P<0.001) at the end of flowering, and was decreased by the addition of PEG (P<0.01).

Discussion

Despite the important role played by forage legumes such as sainfoin in the livestock production systems of a number of countries and growing awareness on their potential for improving protein self-sufficiency, there are few accounts to be found in the literature of experiments focusing on conserved tanniniferous legume forages.

Chemical composition and fermentation profiles

Nitrogen (N) content in the wrapped silage of sainfoin was higher at the early flowering, in accordance with the higher leaves-to-stems ratio (47:53 *versus* 36:64) and the lower cell–wall content (NDF) than at the end of flowering. Scharenberg *et al.* (2007) reported higher N content and lower cell-wall content than here for sainfoin silaged at the early flowering, probably due to the presence of 14.5% of dandelion.

Condensed tannins are considered beneficial for silage fermentation as they protect forage proteins from degradation (Salawu *et al.*, 1999) by inhibiting plant and microbial enzymes and/or by forming complexes with proteins (Waghorn, 2008). Forages containing CT undergo less ensilage-process proteolysis and less transformation of their plant protein nitrogen (N) into NPN compared to forages without CT (Albrecht and Muck, 1991, Fraser *et al.*, 2000). The values we recorded for NH3-N, soluble N and acetic acid in the juice of wrapped silage bales of sainfoin indicated a good-quality conservation according to the Dulphy and Demarquilly

classification (1981). The lower amount of NH3-N, soluble N and acetic acid in the silage prepared at the early flowering from the less mature forage could indicate that the degradation of nitrogen was less extensive due to a higher binding effect of tannins. The NH3-N and soluble N levels (expressed in % N) reported by Dentinho *et al. (*2006) on sulla (*Hedrysarum coronarium L*) silages were lower than in our study (5.85 vs 6.43 for NH3-N and 21.8 vs 40.95 for soluble N, respectively), despite similar N contents. However, Cavallarin *et al.* (2005) found high levels of ammonia (>120g/kg N) in sainfoin silages, possibly due to clostridial activity and enterobacteria, although tannins are known to reduce the activities of silage bacteria and moulds (Guo *et al.*, 2007). Albrecht and Muck (1991) found that the level of non-protein nitrogen in different legume silages was negatively correlated to tannin content, and sainfoin silage showed almost constant values ranging from 350 to 430 g/ kg N, in agreement with our results.

In our study CT activity, at both two phenological stages, was lower in wrapped silage bales compared to the results obtained by Theodoridou *et al* (2010) on the corresponding fresh forages of sainfoin. Moreover, at the same phenological stage (end of flowering) the total CT content (extractable and non-extractable) in wrapped silage bales of sainfoin was lower than the corresponding fresh forage as found previously by Theodoridou *et al* (2010). This contrasts with results obtained by Minnee *et al.* (2002) on *Lotus corniculatus* and *Hedysarum coronarium* and by Scharenberg *et al.* (2007) on sainfoin, who reported that total CT content is not affected by ensiling. However, they reported that the amount of 'free' CTs (CTs not in complex with other molecules) was markedly reduced in the ensiled forages. The remaining CT were 'bound' to other molecules (protein or fibre). This alteration in proportions is believed to be due to the ensiling process causing cell rupture and

release of the CT from the plant cells, allowing them to bind with other molecules (Minnee *et al.* 2002).

In vivo digestibility and nitrogen balance

In vivo digestibility without PEG was lower than found by Demarquilly and Jarrige, (1973) (0.660) but similar to the values reported by Scharenberg *et al*. (2007) (0.608) and Arrigo (2009) (0.637) in sainfoin silages and by Dentinho *et al*. (2006) in sulla haylage (0.596). The *in vivo* digestibility coefficients of wrapped silage bales of sainfoin (0.541 and 0.624, at the end of flowering and at the early flowering, respectively) were lower than the results on corresponding fresh forages (0.607 and 0.654) observed by Theodoridou *et al.* (2010). A review by Demarquilly *et al.* (1998) states that conservation as wrapped silage reduces digestibility due to forage wilt.

In vivo digestibility was not affected by the addition of PEG in the rumen at early flowering stage. This contrasts with Scharenberg *et al.* (2007) who poured a solution of PEG onto sainfoin silage at the early flowering and obtained an increase in digestibility from 0.608 to 0.644.

Tannins decreased total-tract N digestibility from 0.591 at early flowering to 0.548 at end of flowering. These values are in line with Arrigo (2009), who reported a total-tract N digestibility of about 0.60. Fraser *et al.* (2000) on sainfoin silage and Dentinho *et al*. (2006) on sulla haylage found low total-tract N digestibility that led to a negative nitrogen balance. Here, *in vivo* N digestibility in the whole digestive tract was lower for wrapped silage bales of sainfoin than the corresponding fresh forages, (Theodoridou *et al.*, 2009), leading to a higher N quantity in the faeces, despite similar N intake.

The effect of PEG on total-tract N digestibility was significant (P<0.001), thus confirming the presence of active CT in the wrapped silage. The typical effect of CT on the partitioning of N losses was also demonstrated in this study with an increase in faecal N excretion and a decrease in urinary N excretion in presence of active CT, in agreement with Hervas *et al*. (2004) and Waghorn (2008). Our results on N losses fit with those obtained on the same forages studied fresh (Theodoridou *et al.*, 2009). The lower differences between the treatment (with and without PEG) observed for total tract N, digestibility, N in faeces and in urine at the end of flowering were related to lower biological activity and content of CT at this phenological stage.

An important result for animal nutrition was that the N retention expressed in g/g N intake or in g/day/sheep was unaffected by the addition of PEG, and thus by the presence of active CT. As a result of higher N intake and digestibility, N retention was higher for the less mature forage.

In situ dry matter and nitrogen degradability in the rumen

The CT effect on dry matter and nitrogen degradability in the rumen could be evaluated by the addition of PEG. This effect could be explained by the combination of the reduction in the rate of degradation of potentially-degraded material together with the capacity to bind protein or fibre (Aharoni *et al*., 1998). This is confirmed by our results, as PEG affected rate of degradation and effective degradability for DM and N. The DegN of the wrapped silage bales of sainfoin harvested at the early flowering was close to the DegN of fresh sainfoin harvested at vegetative and early-flowering measured by Aufrère *et al*. (2008) (0.591 to 0.625, respectively). In our study DegN measured without PEG at early flowering (0.636) was lower than DegN of silages of other varieties of sainfoin (Ambra: 0.657 and Sepial: 0.696)

(Theodoridou *et al*., 2008) and also lower than that measured on sulla by Dentinho *et al*., (2006) (0.742).

The lower DegN for the early flowering compared to the end of flowering in our study is related to higher CT content at this phenological stage and may be partially explained by the fact that less soluble N was present in this silage.

Rumen fluid composition

The average NH3-N and tN content in the rumen fluid tended to decrease with plant maturity in relation with a lower N content, which was consistent with the results of Kawas *et al.* (1990) and Aufrère *et al.* (2000). Total rumen nitrogen (tN) and ammonia-N (NH3-N) contents remained higher in wrapped silage bales of sainfoin than in corresponding fresh forages (means of 0.24mg/g wrapped silage bales *vs* 0.19mg/g fresh forages) (Theodoridou *et al*., 2009), despite similar forage N contents.

Many papers have suggested that CT contents below 50g/kgDM in the diet do not have important effects on ruminal fermentation (Barry and McNabb, 1999, Salawu *et al.*, 1999). In our study, the CT contents were lower than the recommended values, which vary according to laboratory methods used for CT analysis and may not be applicable for all forages (Mueller-Harvey, 2006).

Makkar *et al.* (1995) and Waghorn and McNabb (2003) explained that high CT contents lead to lower VFA contents in the rumen due to the increase in rumen pool size caused by the slower rate of ruminal digestion. This study recorded a decrease in Deg DM via the presence of CT but no decrease in VFA content. Moreover, acetate-to-propionate ratio was decreased by adding PEG (P<0.01) which is in agreement with Ben Salem (1998) on acacia but in contrast with Yanez Ruiz (2004)

on olive cake. There is literature evidence that the presence of tannins might have a deleterious effect on methanogenic bacteria, which would consequently increase the production of propionate (Sauvant *et al.,* 1995). This may not have been the case in our study.

Conclusion

The results of this experiment indicated that sainfoin preserved as wrapped silage can provide forages that combine good energy and nitrogen value. The CTs in the silage made from sainfoin at early flowering do not reduce the forage digestibility. In sainfoin conserved as wrapped silage, CT decreased total-tract N digestibility but not N retention, which is beneficial for the animal. CTs greatly increased the *in situ* estimates of forage N escaping from the rumen that could be beneficial in terms of flow of dietary nitrogen for animal nutrition. Moreover CTs increased fecal N but decreased N losses in urine. This demonstrates that CT have a direct impact on environmental N emissions as lower urinary urea contents mean less volatile N losses into the environment. Also, faecal N is thought to be an environmentally less harmful form of N as it is more likely to contribute to the accumulation of soil OM than urinary N.

In contrast with tannin-free legumes, sainfoin may be easier to ensile as the presence of CT can prevent protein proteolysis during the ensiling process. Further studies on preserved tanniniferous forages are required in order to identify the mode of action of CT during ensiling and the optimum levels of CT content.

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Table 1 *Chemical composition (g/kg DM), pepsin-cellulase digestibility (Dcellms), content and biological activity of condensed tannins (CT) in wrapped silage bales of sainfoin harvested at two phenological stages (Phs): end of flowering and early flowering.*

	Silage 1	Silage 2	n	s.e.	*P* Phs
Dry matter (DM) (g/kg wrapped big bale)	375	398	8	3.9	*
N (g/kg DM)	22.4	27.8	8	0.56	**
NDF (g/kg DM)	463	393	8	2.83	***
ADF (g/kg DM)	350	310	8	2.53	***
ADL (g/kg DM)	86	68	8	2.10	**
NDIN (g/g N)	0.142	0.107	8	0.001	***
ADIN (g/g N)	0.088	0.068	8	0.001	**
Dcellms (g/g)	0.567	0.635	8	0.001	***
CT-RDA *(g tannic acid equivalent/100gDM)*	0.78	1.10	8	0.05	*
Content HCl-butanol (g/kg DM)	17.6	33.9	8	1.1	**

Silage 1: First vegetation cycle (End of flowering) and Silage 2: Second vegetation cycle (Early flowering), Nitrogen (N), neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL), detergent insoluble nitrogen (NDIN), acid detergent insoluble nitrogen (ADIN), pepsin-cellulase digestibility (Dcellms), condensed tannins (CT); Probability of significant effects due to phenological stage (Phs) in the analysis for wrapped silage bale sainfoin. *P<0.05; **P<0.01; ***P<0.001; ns = non-significant.

Table 2 *Ensiling characteristics of wrapped silage bales of sainfoin harvested at two phenological stages (Phs): end of flowering and early flowering.*

	Silage 1	Silage2	n	s.e.	*P* Phs
pH	4.91	4.61	8	0.03	**
NH3-N (% N)	7.87	3.82	8	0.32	***
Soluble N (% N)	45.81	36.09	8	0.82	**
Acids (g /kgDM)					
Lactic	22.76	28.87	8	1.74	ns
Acetic	6.83	5.68	8	0.31	ns
Propionic	0.18	0.03	8	0.02	**
Butyric	0.83	0.07	8	0.05	***
Alcohols (g /kgDM)					
Methanol	0.38	1.16	8	0.05	***
Ethanol	1.88	1.77	8	0.18	ns

Silage 1: First vegetation cycle (end of flowering) and Silage 2: Second vegetation cycle (early flowering), DM: dry matter, NH3-N: ammonia nitrogen, N: total nitrogen. Probability of significant effects due to phenological stage (Phs) in the analysis for wrapped big bale sainfoin. *P<0.05; **P<0.01; ***P<0.001; ns = non-significant.

Table 3 *Total-tract organic matter (OM) and nitrogen (N) digestibility, N losses and N retention for sheep fed wrapped silage 1 and silage 2 with or without addition of polyethylene glycol (PEG) in the rumen (n=6 for each treatment)*.

	Silage 1		Silage 2		s.e.	T	Phs	T×Phs
PEG treatment	**with**	**without**	**with**	**without**				
N intake (g/d per sheep)	33.76	30.12	43.24	40.31	1.45	ns	***	ns
Digestibility								
OM	0.567	0.541	0.627	0.624	0.006	ns	***	*
Total-tract N digestibility	0.655	0.548	0.737	0.591	0.011	***	***	*
N (g/gNintake)								
In faeces	0.346	0.442	0.261	0.408	0.010	***	**	ns
In urine	0.417	0.400	0.455	0.321	0.015	*	ns	***
Nitrogen retained								
g/d/ sheep	7.66	4.93	12.12	11.22	0.82	ns	***	ns
g/g N intake	0.23	0.16	0.28	0.27	0.02	ns	***	*

Silage 1: First vegetation cycle (end of flowering) and Silage 2: Second vegetation cycle (early flowering). Probability of significant effects due to the treatment (T), phenological stage (Phs) and their interaction (T×Phs) in the analysis for wrapped big bale sainfoin. *P<0.05; **P<0.01; ***P<0.001; ns = non-significant.

Table 4 *Mean content of total nitrogen (tN) (mg/g), NH3-N (mg/g) and volatile fatty acids (mmol/L) in the rumen fluid of sheep fed wrapped big bale sainfoin at the first vegetation cycle (end of flowering) and at the second vegetation cycle (early flowering) with or without addition of polyethylene glycol (PEG) in the rumen.*

	Silage 1		Silage 2		s.e.	T	Time	Phs	T×Phs
PEG treatment	**with**	**without**	**with**	**without**					
Total Nitrogen (tN)	0.31	0.28	0.37	0.32	0.02	ns	***	***	ns
Ammonia-Nitrogen (NH3-N)	0.21	0.21	0.25	0.23	0.01	ns	***	***	ns
Volatile fatty acids (VFA)	90.5	92.2	102.1	97.5	2.3	ns	***	***	ns
Acetate:Proprionate ratio	3.67	4.07	3.44	3.90	0.09	**	***	***	ns

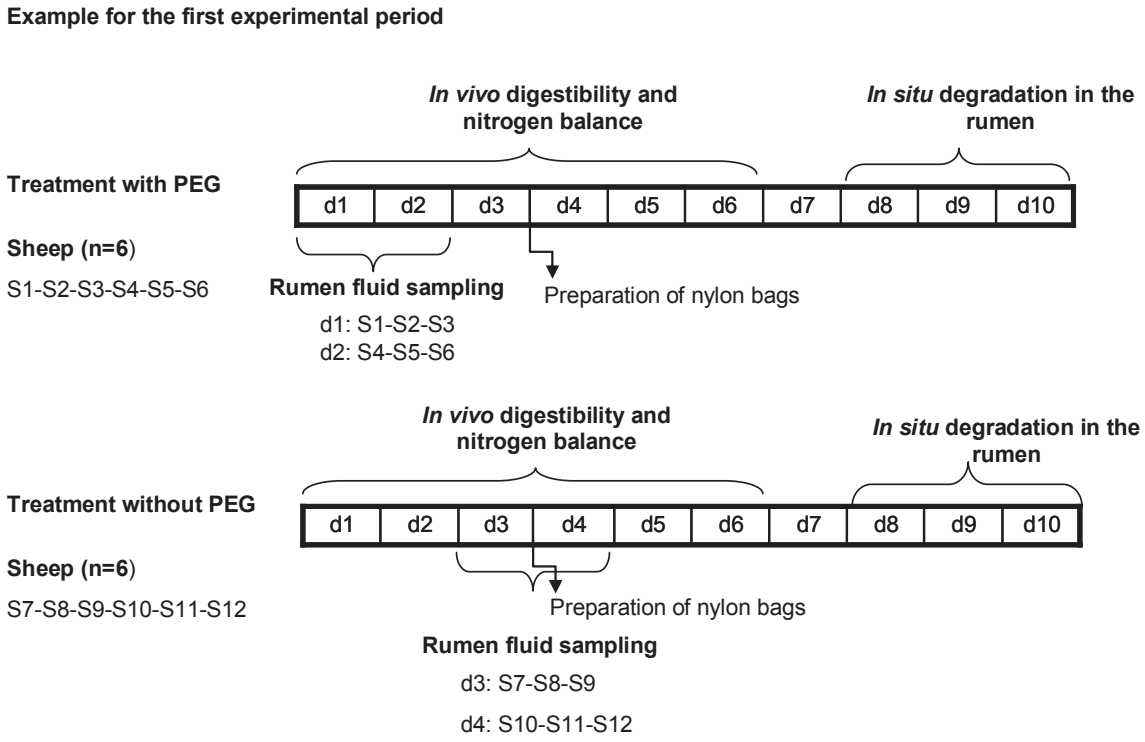
Silage 1: First vegetation cycle (end of flowering) and Silage 2: Second vegetation cycle (early flowering). Probability of significant effects due to treatment (T), phenological stage (Phs) and their interaction (T×Phs) in the analysis for wrapped big bale sainfoin. *P<0.05; **P<0.01; ***P<0.001; ns = non-significant.

Table 5 *In situ rumen degradability of dry matter (DegDM) and nitrogen (DegN) of sainfoin studied as wrapped big bale at the first vegetation cycle (end of flowering) and at the second vegetation cycle (early flowering 5 weeks after the end of flowering) with or without addition of polyethylene glycol (PEG) in the rumen.*

	Silage 1		Silage 2		s.e.	T	Phs	T×Phs
PEG treatment	**with**	**without**	**with**	**without**				
Dry matter degradability								
a	0.272	0.271	0.262	0.271	0.002	ns	ns	ns
b	0.430	0.440	0.479	0.534	0.010	ns	**	ns
c	0.086	0.072	0.079	0.046	0.003	***	*	*
DegDM	0.525	0.510	0.536	0.495	0.004	**	ns	ns
Nitrogen degradability								
a	0.400	0.398	0.327	0.340	0.004	ns	***	ns
b	0.463	0.422	0.572	0.602	0.023	ns	**	ns
c	0.147	0.102	0.093	0.058	0.003	***	***	ns
DegN	0.727	0.664	0.678	0.636	0.004	***	***	ns

Silage 1: First vegetation cycle (end of flowering) and Silage 2: Second vegetation cycle (early flowering), a: rapidly degradable fraction, b: slowly degradable fraction, c: degradation rate of fraction b (h^{-1}), DegDM or DegN: *in situ* ruminal degradability of DM or N: *in situ* ruminal degradability of DM or N calculated with a passage rate of 0.06/h; Probability of significant effects due to treatment (T), phenological stage (Phs) and their interaction (T×Phs) in the analysis for wrapped big bale sainfoin. *P< 0.05; **P<0.01; ***P<0.001; ns = non-significant.

Figure 1 Timeline of the experimental design for the *in vivo* and *in situ* measurements.



At the first experimental period:

S1-S2-S3-S7-S8-S9: offered the wrapped silage bales of sainfoin at the first vegetation cycle (end of flowering)

S4-S5-S6-S10-S11-S12: offered the wrapped silage bales of sainfoin at the second vegetation cycle (early flowering)

At the second experimental period:

S1-S2-S3-S7-S8-S9: offered the wrapped silage bales of sainfoin at the second vegetation cycle (early flowering)

S4-S5-S6-S10-S11-S12: offered the wrapped silage bales of sainfoin at the first vegetation cycle (end of flowering)

Figure 2 Evolution of the content (mg/g) of total nitrogen (tN) in the rumen fluid for silage 1 and silage 2 with or without addition of polyethylene glycol (PEG) in the rumen, before the morning meal (0 h) and 1.5 h, 3 h and 6 h after the meal.

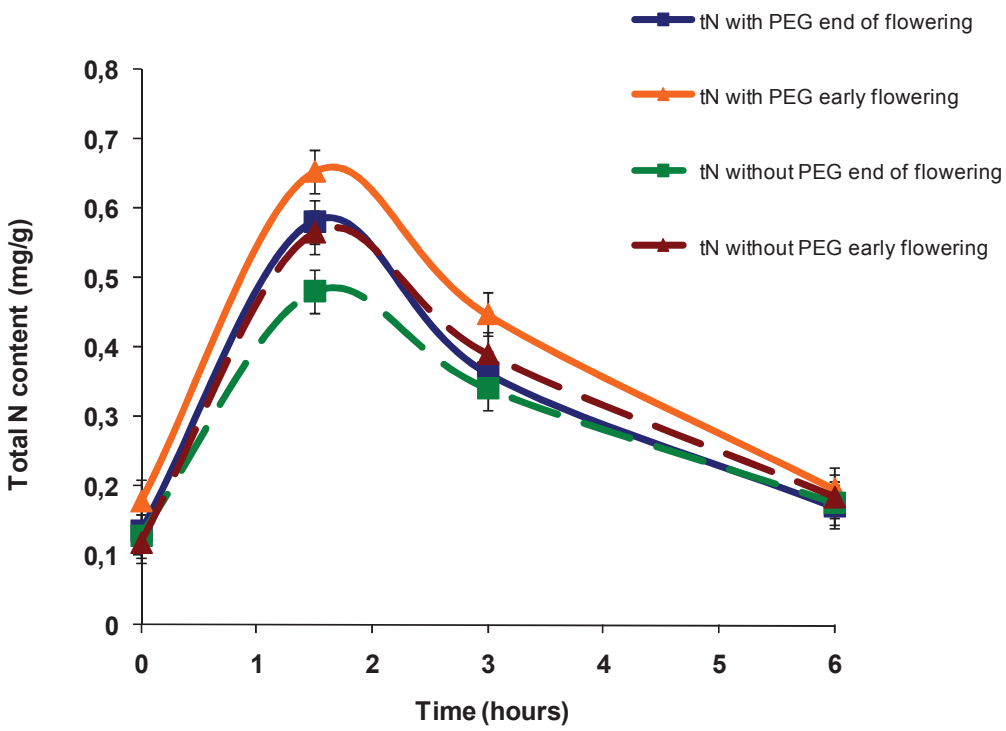
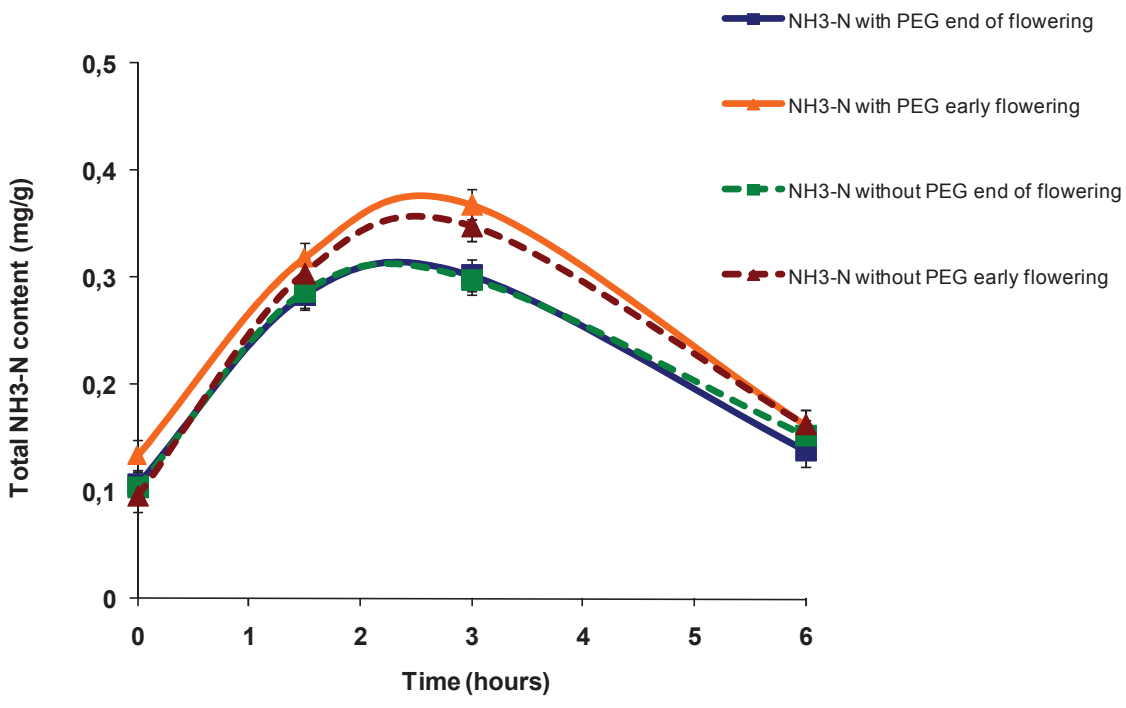


Figure 3 Evolution of the content (mg/g) of ammonia-N (NH3-N) in the rumen fluid for silage 1 and silage 2 with or without addition of polyethylene glycol (PEG) in the rumen, before the morning meal (0 h) and 1.5 h, 3 h and 6 h after the meal.



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PART 4

Evaluation of sainfoin varieties in term of their chemical composition, content, structure and biological activity of condensed tannins. Comparison with lucerne.

Submitted to *Grass and Forage Science*

Effect of plant development during first and second growth cycle on chemical composition, condensed tannins and nutritive value of three sainfoin (*Onobrychis viciifolia*) varieties and lucerne.

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Abstract

Sainfoin (*Onobrychis viciifolia*) is a temperate legume which contains condensed tannins (CT), i.e. polyphenols that are able to bind proteins and thus reduce protein degradation in the rumen. Few studies exist that investigated the relation between plant phenology, CT characteristics and nutritive value of sainfoin. Plants were harvested on 9 dates in the first and 4 dates in the second growth cycle. Phenological stages were analysed for leaf-to-whole-plant ratio, nitrogen (N), organic matter digestibility (OMd) by the pepsin-cellulase method, cell-wall concentration, N solubility (solN) plus CT concentration, biological activity and structures. Three sainfoin varieties (Esparcette, Ambra, Villahoz) were compared with a tannin-free legume, lucerne (Aubigny). Leaf-to-whole-plant, cell-wall concentration, N of

sainfoin and lucerne were closely related with the phenological stage. Although sainfoin developed earlier than lucerne, its pepsin-cellulase digestibility was higher or close to lucerne. Esparcette had the lowest OMd, N and solN and the highest CT concentration. CT concentration, proportion of prodelphinidins (PD) and mean degree of polymerisation (mDP) increased with the phenological stage (or plant maturity) and these parameters were associated with lower biological activity of CT. Sainfoin varieties differed in their relationships between phenological stages, nutritional parameters and CT characteristics. The results demonstrated that sainfoin can be a valuable alternative forage legume to lucerne.

Keywords: forage legume, chemical composition, condensed tannin contents and composition, digestibility, phenological stage

Introduction

There is renewed interest in forage legumes because they contribute to sustainable feeding systems; they are able to fix nitrogen and have a high feed value for ruminants. However, ruminants make poor use of forage legume proteins, as these are often rapidly degraded in the rumen and this leads to large urinary N losses (Min *et al.*, 2003). The use of tanniniferous legumes is one solution for improving the protein utilisation by ruminants. Protein degradation in the rumen is reduced in the presence of condensed tannins (CT) (Waghorn, 2008). Whilst high tannin concentrations can reduce voluntary feed intake and nutrient digestibility (Waghorn *et al.,* 1999), low to moderate concentration can improve feed utilisation. A reduction in protein degradation in the rumen can lead to a subsequent increase in amino acid flow to the small intestine (Frutos *et al.,* 2004). Sainfoin is a temperate tanniniferous legume. Marked differences among sainfoin varieties appear to exist in terms of agronomic parameters (Hayot-Carbonero *et al.*, 2011). However, little is known about their nutritive value, tannin concentrations and structures. In fact, plant breeding has paid little

attention to improving sainfoin compared to other forage legumes such as lucerne. According to Hayot-Carbonero *et al*. (2011) geographic origin is one of the most important sources of genetic variability. Moreover, few studies have investigated the relation between plant phenology (McGraw and Marten 1986; Borreani *et al.*, 2003), CT composition and nutritive value of sainfoin. Identifying the most appropriate growth stage is crucial to optimising harvesting, grazing and thus forage utilisation. Forages from the first growth cycle are mainly used as silage or hay because of high yields; whereas young forages from the regrowth (second growth cycle) tend to be used for grazing (Butler *et al.,* 2002).

This study was designed to investigate differences among three sainfoin varieties, which originated from different geographical areas and which were cultivated at the same site over the course of two growth cycles. Changes of phenology, leaf-to-whole-plant ratio, chemical composition and nutritive value were analysed and related to CT composition. Lucerne was used as a non-CT-containing legume and was cultivated in the same site and compared with the sainfoin varieties.

Material and methods

Three varieties of sainfoin (*Onobrychis viciifolia*) (Esparcette from Ukraine, Ambra from France and Villahoz from Spain) were studied in comparison with lucerne (var. Aubigny). They were sown in August 2008 and cultivated on the INRA Clermont-Ferrand-Theix site (France) at an altitude of 870 m on a granitic brown soil in a full factorial design with three replicate blocks. Samples of the three replicates were harvested at 6 cm above ground level on 9 different dates in 2009 during the first growth cycle and at 4 different dates during the second growth cycle.

For each harvest date, we evaluated the phenological stage and the morphological composition (leaf-to-whole-plant ratio) for each sainfoin variety and lucerne using the classification system of Borreani *et al.* (2003). This was modified for our study as described in Table 1. For each species, 50 tillers were collected at random and classified according to stage (Table 1). The mean growth stage (MSW) was then calculated using the following equation:

$$\text{MSW}=\sum \frac{Si \times Di}{W}$$

where:

Si =phenological stage number, 0 to 9, Di = dry weight of the plants in stage Si, and W=total dry weight of the plants at all stages.

The herbage was divided into leaves and stems (flowers were included in the stem fraction). The developmental stage in each species was studied according to the change of the sum of positive average daily temperatures from 1st February (growing degree day, °C d) for the first growth cycle and from the harvest date of 5 May for the second growth cycle. The samples were harvested during the first growth cycle on 30 April (409 °C d), 5 May (456°C d), 12 May (554°C d), 19 May (635°C d), 26 May (752°C d), 2 June (836°C d), 9 June (921°C d) 15 June (1013°C d) and 23 June (1122°C d). The samples were harvested during the second growth cycle, i.e. 4 weeks after cutting on 2 June (379°C d), 9 June (464°C d) 15 June (570°C d) and 23 June (679°C d).

Laboratory analyses

The laboratory analyses were performed on whole plant samples. One half of all samples were dried at 60° C in a forced–air oven; the other half was freeze-dried. Samples were ground to

pass a 1 mm sieve. N was determined by the Kjeldahl method (AOAC 1990, method 977.02). Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin (ADL) were determined using the ANKOM filter bag method (ANKOM Technology, Macedon, New York , USA) according to Van Soest and Wine (1967) modified by Pagan *et al.* (2009). Pepsin-cellulase digestibility was determined according to Aufrère and Michalet-Doreau (1983). Freeze-dried samples were analysed directly for condensed tannins (CT) by thiolysis with benzyl mercaptan at 40°C for 1 h (Gea *et al.,* 2011). Tannin content included extractable plus unextractable CT; tannin composition was described by mean degree of polymerisation (mDP) (i.e., molecular size), prodelphinidin:procyanidin (PD:PC; i.e., % PD content) and *cis*:*trans* (i.e. % *cis* content) ratios (Gea *et al.,* 2011). The biological activity of CT in terms of protein-binding capacity was determined using the radial diffusion assay as described by Makkar *et al.* (2000). This assay measured the precipitation of bovine serum albumin, where the area of the precipitation ring is proportional to the amount of extracted CT.

These parameters were also determined by near infrared spectroscopy (NIRS) according to the following procedure. All samples were scanned in a monochromator NIRsystems 6500, but laboratory determinations were performed on a subset of 37 samples which had been selected based on their spectral information (Shenk and Westerhaus, 1994). These laboratory analyses were then used with the NIR spectra in order to expand the prediction equations for routine analysis. The statistical parameters (standard error of cross validation and coefficient of determination) of the new prediction models were as follows: 0.08 and 0.99 for N (g kg^{-1} DM); 1.59 and 0.97 for NDF (g kg^{-1} DM); 0.99 and 0.98 for ADF (g kg^{-1} DM); 0.43 and 0.95 for ADL (g kg^{-1} DM), 1.49 and 0.98 for pepsin-cellulase digestibility (g/g DM); 0.13 and 0.86 for CT (g kg^{-1} DM); 7.07 and 0.55 for mDP; 3.91 and 0.65 for PD (%) and 4.03 and 0.47 for the *cis* flavanol content (%).

Freeze-dried samples were used to determine nitrogen solubility (solN) in artificial saliva buffer (expressed in g/100g N) was performed in the presence or absence of PEG (polyethylene glycol 4000) in order to assess tannin effects, as PEG binds with tannins and thus prevents them from binding with proteins. Freeze-dried samples (0.7 g of dry matter basis) were incubated with 50 ml artificial saliva for one hour; in half of these samples PEG 4000 was added (600 mg) to inhibit the CT effect in sainfoin.

Statistical analysis

For chemical composition (N, NDF, ADF, ADL), condensed tannin concentration, biological activity, mean degree of polymerisation (mDP), prodelphinidins (PD), c*is* flavanol content, leaf-to-whole-plant ratio and phenological stage data were analyzed using the Mixed Procedure of the SAS software package (2000) and were submitted to ANOVA testing of species, cycle variety and sampling date as fixed effects. Sampling date was considered as repeated variable.

For solN, data were submitted to ANOVA for testing of variety, date and treatment (T) (with or without PEG) as fixed effects.

The relationship between vegetation characteristics (phenological stage and leaf-to-whole plant ratio), chemical composition and pepsin-cellulase digestibility was evaluated by using Pearson correlation coefficients.

Results

Phenological stage

From the beginning of the first growth cycle, the phenological development of the three varieties of sainfoin was significantly more advanced than that of lucerne, but occurred at a

similar rate for sainfoin and lucerne (Figure 1a). However Ambra (3.64) developed more rapidly than Esparcette and Villahoz (2.28 and 2.70, respectively) at the beginning of first growth cycle but Villahoz reached a more advanced mean growth stage than Esparcette and Ambra at the end of the cycle (Figure 1a).

During the second growth cycle, the phenological development of sainfoin was also significantly more advanced than that of lucerne. It ranged from 4.52 (early bud) to 5.50 (late bud) for the sainfoin varieties (Table 2b) and from 1.0 (early vegetative) to 4.58 (early bud) for lucerne (Figure 1a). However, the rate of development of lucerne (1.00 to 4.58) was significantly higher than that for sainfoin (4.52 to 5.50) (species×date, $P<0.001$) (Figure 1a).

During the first growth cycle, a high correlation was observed between phenological stage and N (r=-0.85), whereas the same correlation during the second vegetation cyle was r =-0.85).

Leaf-to-whole-plant ratio

Leaf-to-whole-plant ratio decreased during the first growth cycle from 0.79 to 0.23 for sainfoin and from 0.52 to 0.34 for lucerne. The leaf-to-whole-plant ratio was significantly higher for sainfoin than for lucerne at the beginning of the cycle, but declined rapidly until 26 May (from 0.79 to 0.35) and more slowly thereafter (from 0.35 to 0.23). Thus the leaf-to-whole-plant ratio was significantly higher for lucerne at the end of the cycle. Among the sainfoin varieties, Ambra and Villahoz showed the same trend, but Esparcette had a significantly higher leaf-to-whole-plant ratio before 26 May and a lower leaf-to-whole-plant ratio thereafter (Figure 1b).

During the second growth cycle, the leaf-to-whole-plant ratio declined more rapidly in sainfoin than lucerne (species×date, $P<0.001$, Table 2b). In contrast to the first growth cycle,

Esparcette had a higher leaf-to-whole-plant ratio than the other two varieties (Figure 1b and Table 2b). Lucerne had a higher leaf-to-whole-plant ratio on 23 June than sainfoin (P<0.001).

A high correlation was found between leaf-to-whole-plant ratio and N (r=0.94) during the first and also the second growth cycle (r=0.97).

Chemical composition

During the first and second growth cycles, sainfoin varieties exhibited a similar pattern for N content and leaf-to-whole-plant-ratio. During the first growth cycle, N decreased rapidly from 38.0 g to 18.9 g kg^{-1} DM by 26 May and remained rather constant thereafter (Figure 2a). In the second growth cycle, the rate of N decrease was constant between 2 and 23 June. Lucerne had significantly slower N decrease (species×date P<0.001) and N content remained significantly higher at the end of the cycle (Table 2a). Among the sainfoin varieties, Esparcette had a lower N content at the beginning of the 1st cycle, but sainfoin varieties showed no differences in the second cycle.

In the absence of PEG, lucerne had significantly higher soluble nitrogen concentration (solN, g/100g N) than sainfoin (Figure 2b). However, in the presence of PEG, sainfoin had significantly higher solN concentration, which was similar to lucerne for first and second growth cycles, respectively.

Sainfoin varieties differed significantly in solN (PEG treatment). Esparcette had the lowest and Villahoz the highest value (Figure 2b).

Esparcette had the highest ADF and NDF concentrations in the first and the lowest in the second growth cycle. Lucerne and sainfoin showed similar trends of NDF concentrations, but lucerne NDF was significantly higher in both growth cycles (Figure 3a). ADL followed the

NDF and ADF trends in both growth cycles. Lucerne also had higher ADL concentrations (Figure 3c).

Pepsin-cellulase digestibility

During the first growth cycle, sainfoin had significantly higher pepsin-cellulase digestibility than lucerne (0.694 *vs* 0.670, Figure 4 and Table 2a) with the exception of Esparcette. Pepsin-cellulase digestibility decreased, as harvest date increased; (species×date, $P<0.01$) and sainfoin varieties (var×date, $P<0.001$) revealed different trends (Figure 4). The pepsin-cellulase digestibility of Esparcette decreased more rapidly and had the lowest digestibility at the end of the cycle.

During the first growth cycle, our data on sainfoin revealed a high correlation between pepsin-cellulase digestibility and phenological stage or leaf-to-whole-plant ratio (r= -0.85and r=0.93, respectively). In contrast, during the second growth cycle, pepsin-cellulase digestibility did not differ between species or varieties (Table 2b). A high correlation was also observed between pepsin-cellulase digestibility and phenological stage or leaf-to-whole-plant ratio during the second growth cycle (r = -0.89 and 0.95, respectively).

Condensed tannins in sainfoin

CT concentration

During the first growth cycle, the mean CT concentration increased from 3.66 to 6.40 g kg^{-1} DM until 26 May and then decreased slowly until reaching 5.7 g kg^{-1} DM on 23 June (Figure 5a). All three varieties showed the same trend. CT concentrations did not vary with harvest date in the second growth cycle.

For the first growth cycle, CT concentration was significantly higher in Esparcette (6.05 g kg^{-1} DM) than Villahoz (4.25 g kg^{-1} DM) and Ambra (5.53 g kg^{-1} DM) (P<0.001, Table 3a). The same differences between varieties were observed during the second growth cycle (Table 3b).

Mean degree of polymerisation (mDP)

During the first growth cycle, the mDP of CT increased progressively from 19 May to the last harvest date (P<0.001) and varied among the three varieties (P<0.001) (Figure 5b and Table 3a). During the second growth cycle, the mDP increased also with harvest date, but did not vary between varieties (P>0.05) (Table 3b).

Proportion of prodelphinidin (PD) tannins

In line with the mDP during the first growth cycle, the PD proportion increased from 19 May and reached a maximum of 74.2 on 23 June. PD varied between the three varieties in the first but not the second cycle (Figure 5c).

Proportion of cis flavanols

During the first growth cycle, the proportion of *cis* flavanols increased from 19 May until 23 June and varied among the three varieties (Figure 5d and Table 3a). Esparcette had the highest *cis* proportion in both cycles (Table 3b).

Biological activity

CT biological activity decreased as the harvest date increased in both growth cycles and all varieties (Figure 5e). Villahoz had the lowest and Esparcette the highest biological activity (Table 3a) in both cycles (P<0.05).

Discussion

This study provides detailed information on the chemical composition, digestibility, CT content and composition of three sainfoin varieties. These parameters were related to their morphological development over two growth cycles and compared to lucerne.

Plant development and chemical composition

The phenological stage and the leaf-to-whole-plant ratio proved useful for understanding changes in chemical composition. The higher mean N value in lucerne than in sainfoin agrees with data by McGraw and Marten (1986) and can be related to the less advanced phenological development of lucerne. At the end of the first growth cycle, the higher leaf proportion in lucerne than sainfoin also contributed to its higher N as leaves contain more nitrogen than stems (Borreani *et al.*, 2003). Similar processes can also explain the higher N in lucerne during the second growth cycle. N decreased faster with phenological stage in sainfoin than lucerne, probably because of a more rapid morphological change in leaf-to-stem ratio, which started at the beginning of flowering (Borreani *et al.*, 2003). In agreement with our results, Sheaffer *et al.* (2000) reported decreasing N and increasing fibre concentrations with changes in lucerne leaf-to-whole-plant ratios due to advancing maturity. Although sainfoin developed earlier than lucerne, the cell-wall concentration in sainfoin was lower or similar to lucerne, and depended on variety.

Phenological and morphological development can also help to understand differences between the sainfoin varieties. Esparcette in particular behaved differently from the other two varieties. During the first growth cycle, a lower leaf-to-whole-plant ratio explained its lower N and higher cell-wall concentrations. However, Esparcette had a higher leaf-to-whole-plant

ratio in the second growth cycle, which explained its lower NDF (P<0.001), ADF (P<0.001), ADL (P<0.05) concentrations.

Digestibility

Sainfoin had similar or higher pepsin-cellulase digestibility than lucerne and this confirmed previous results of Aufrère *et al*. (2008). They studied *in vivo* digestibility of sainfoin at vegetative and early flowering stages and lucerne digestibility at vegetative and bud flowering stages and found no differences between the two species.

Comparing the same phenological stages, lucerne had higher pepsin-cellulase digestibility in the second than the first growth cycle. These results agreed with findings of Sheaffer *et al.* (1998) who reported lower forage quality of lucerne during the first than subsequent harvests at the same maturity. In our study, the better lucerne quality found in the second growth cycle agreed with the higher leaf-to-whole-plant ratio at the same phenological stage. A similar phenomenon was observed for Esparcette. This sainfoin variety showed the most contrasting leaf-to-whole-plant ratios between the two growth cycles. Thus, the decrease in pepsin-cellulase digestibility of sainfoin and lucerne with increasing maturity was linked to increases in NDF and ADF concentrations. This was clearly a combined effect of decreasing leaf and increasing stem proportions. Among sainfoin varieties, the most striking difference was the fast digestibility decrease of Esparcette during the first growth cycle.

Condensed tannins

Lucerne does not contain CT and therefore PEG addition had no effect on its solN. The solN of sainfoin was markedly lower but increased to a similar level after PEG addition (Makkar, 2003). This indicates the presence of active CT in sainfoin, which lower protein degradation in the rumen and this can lead to a better protein utilisation by ruminants (McNabb *et al.*,

1996; Theodoridou *et al.*, 2010). Important differences in nitrogen solubility between sainfoin varieties were observed and were consistent with CT concentration and biological activity. Esparcette had the lowest solN, the highest CT concentration and biological activity during both cycles. In contrast, Villahoz had the highest solN and lower CT concentration and biological activity.

Despite several studies on *Lotus* CT concentrations and structures (Hedqvist *et al.,* 2000; Min *et al.,* 2003; Meagher *et al.,* 2004), few reports have analysed sainfoin CT and their changes with phenological stages. Sainfoin CT are known to be located mainly in the leaf tissue (Lees *et al.,* 1993). Thus, an unexpected result was that the CT concentration tended to increase with the phenological stage for both growth cycles although the leaf-to-whole-plant ratio decreased. In contrast to previous results on sainfoin (Borreani *et al.*, 2003) and sulla (Piluzza *et al.*, 2000) , we observed that the CT concentrations increased in all three sainfoin varieties from 19 May, which corresponded to the end of the vegetative phase of the first growth cycle. During their growth period, when plants produce a lot of biomass, few resources are available for synthesing phenolic compounds. However, during flowering, when growth is reduced, excess carbon may be available for tannin synthesis (Iason *et al.,* 1993). So the development of flowers, which are rich in CT (Gebrehiwot *et al.*, 2002) began after 19 May and might explain the increase in CT concentration for the three sainfoin varieties. According to Mole *et al.* (1988), CT concentrations vary also with the light intensity, soil fertility (Lascano *et al.,* 2001), temperature and plant maturity. In the second growth cycle, the photoperiod was longer, which may explain the higher development rate of sainfoin. Mean daily temperature was also higher in the second (13.7°C) compared to the first cycle (9.5°C from 1 April). Lees *et al.* (1994) reported that *Lotus pedunculatus* had more CT when when grown at 30°C than 20°C. Similarly, Anuraga *et al.* (1993) observed higher CT levels in *Lotus pedunculatus* but

not in *Lotus corniculatus* at 30°C. Thus, environmental factors could explain the higher sainfoin CT concentration and biological activity in the second cycle.

The variations of the biological activity of CT and CT concentrations were positively related amongst sainfoin varieties and growth cycles. However, within a growth cycle as phenological stage increased, the biological activity of CT decreased together with the leaf-to-whole-plant ratio despite the fact that total CT concentrations increased. This could indicate that the biological activity of CT may differ between leaves, flowers and stems (Gebrehiwot *et al.*, 2002). An alternative explanation could come from the fact that the thiolysis method measured extractable and unextractable CT (Gea *et al.*, 2011), whereas the biological activity method was based on extractable CT. A likely explanation could be that a decrease of biological activity with increasing maturity resulted from lower proportions of extractable CT. In fact, Gea *et al.* (2011) showed that larger tannins, i.e. tannins with higher mDP, were more difficult to extract than smaller tannins.

Mueller-Harvey (2006) suggested that CT properties depend on their structures. CT vary in terms of their monomer units, which gives rise to a wide range of PD:PC and *cis:trans* ratios, their mean degrees of polymerisation (mDP) and the linkages between the flavan-3-ol units (Meagher *et al*., 2004). Our study found an increase in % PD with the phenological stage in contrast with previous results (Theodoridou *et al.*, 2010; Guimaraes-Beelen *et al.*, 2006), where % PD in whole plants hardly changed with phenological stage. The whole plant % PD contents reported here (67 to 71%) are comparable with % PD of extractable tannins (Koupai-Abyazani *et al.* 1993b; Gea *et al*., 2011), which ranged from 55 to 85% for several different sainfoin accessions.

According to Frazier *et al*. (2010) and Sarni-Machado *et al*. (1999) polymer composition affects CT properties. Prodelphinidins have three phenolic groups unlike procyanidins which

have only two phenolic groups in the B-ring; and this may explain why higher PD proportions promoted complex formation between CT and proteins (Guimaraes-Beelen *et al*., 2006). This relationship was not observed, however, in our study as the astringency of tannins, or their protein binding capacity, may be related not only to the proportion of PD but also to the spatial distribution of these monomers within the tannin molecule (Rosales, 1999). Moreover, Koupai-Abyazani *et al*. (1993a) showed that the degree of polymerisation of CT changed during sainfoin tissue maturation. Similarly, in maturing oak leaves, CT concentration, degree of polymerisation and astringency increased (Makkar *et al*., 1991). The present study also found a parellel increase of mDP and CT concentration with phenological stage during the first growth cycle (Figure 5). Bell *et al.* (1992) reported that the CT concentration increased progressively in each new leaf of cotton (*Gossypium* spp.) until plateau concentrations were reached in leaves, which had developed during onset of fruiting.

The present study found that the proportion of *cis* flavanols increased with the phenological stage during the first growth cycle, whereas the biological activity decreased. These results could be explained by the observation of De Freitas and Mateus (2001) who studied synthesised CT dimers and trimers. They found that the number of active sites that were able to bind proteins increased with the number of *cis* flavanol units. To date the literature contains very little information on the role of the *cis*/*trans* ratio in terms of the biological activity of CT.

Conclusion

This study investigated CT characteristics in relation to plant development of three sainfoin varieties and lucerne. Sainfoin showed a faster development rate than lucerne during both growth cycles but differences were also observed between sainfoin varieties. Sainfoin CT

contents had no negative effect on digestibility as measured by the pepsin-cellulase method. Variability due to the geographical origin of sainfoin varieties played an important role for the digestibility during the first growth cycle. Esparcette had the highest CT concentration, *cis* flavanol content and biological activity and this could explain its lowest N solubility in the second growth cycle. The presence of CT reduced protein solubility in the rumen and could make sainfoin useful as an alternative forage to lucerne or grasses from a nutritive and environmental point of view. Results demonstrated that more attention should be paid to CT concentration and structure. The differences among varieties need to be evaluated in *in vivo*.

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Table 1 Stage numbers for the phenological stages of sainfoin and lucerne

Stage number	Phenological stage name	Stage definition
1	Early vegetative	Stem length<20cm
2	Mid vegetative	Stem length<30cm; no buds or flowers
3	Late vegetative	Stem length>30cm; no buds or flowers
4	Late vegetative	Stem length>45cm; no buds or flowers
5	Early bud	1 to 2 floral buds; no open flowers
6	Late bud	>2 floral buds; no open flowers
7	Early flower	1 node with open flowers
8	Late flower	Open flowers; no green pods
9	Early seed pod	Brown flowers; 1st green pods

Table 2a) Leaf-to-whole-plant ratio (g g^{-1}), phenological stage number, nitrogen concentration (N) (g kg^{-1} DM), neutral detergent fiber (NDF) (g kg^{-1}DM), acid detergent fiber (ADF (g kg^{-1}DM)), acid detergent lignin (ADL) (g kg^{-1}DM), pepsin-cellulase digestibility (g g^{-1}) of three sainfoin varieties at different dates during the first growth cycle.

First growth cycle															
	Harvest Date														
	30 April	5 May	12 May	19 May	26 May	2 June	9 June	16 June	23 June	s.e.	Sp	Var	Date	Sp×Date	Var×Date
Phenological stage	2.02	1.81	2.87	3.63	4.27	5.04	5.34	6.01	6.53	0.143	***	***	***	NS	***
Leaves/Whole plant	0.79	0.72	0.57	0.46	0.35	0.32	0.26	0.25	0.23	0.151	**	**	***	***	**
Total N	38.2	25.7	38.0	24.7	17.7	15.7	14.5	14.4	14.7	1.20	**	***	***	***	*
NDF	218	208	245	297	398	443	448	473	475	9.4	**	***	***	**	*
ADF	152	152	182	229	308	345	343	363	366	7.7	**	***	***	*	**
ADL	27	21	26	38	57	64	68	76	74	2.1	***	***	***	**	**
Pepsin-cellulase digestibility	0.79	0.83	0.79	0.75	0.67	0.63	0.62	0.58	0.58	0.009	***	***	***	**	***

Species (Sp), variety (Var), s.e. standard error of means, NS, non significant, *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$.

Table 2 b) Leaf-to-whole-plant ratio (g g^{-1}), phenological stage number, nitrogen concentration (N) (g kg^{-1} DM), neutral detergent fiber (NDF) (g kg^{-1}DM), acid detergent fiber (ADF) (g kg^{-1}DM), acid detergent lignin (ADL) (g kg^{-1}DM), pepsin-cellulase digestibility (g g^{-1}) of three sainfoin varieties at different dates during the second growth cycle.

Second growth cycle										
	Harvest Date									
	2 June	9 June	16 June	23 June	s.e.	Sp	Var	Date	Sp*Date	Var*date
Phenological stage	4.52	4.80	5.27	5.50	0.146	***	***	***	***	NS
Leaf/Whole plant	0.59	0.47	0.38	0.23	0.22	NS	***	***	***	**
Total N	28.6	25.3	21.1.	18.2	0.95	***	NS	***	NS	NS
NDF	263	291	345	385	8.7	***	***	***	NS	**
ADF	195	217	259	291	7.3	***	***	***	*	*
ADL	32	37	49	54	1.9	***	*	***	NS	NS
Pepsin-cellulase digestibility	0.78	0.76	0.70	0.67	0.009	NS	NS	***	**	NS

Species (Sp), variety (Var), s.e. standard error of means, NS, non significant, *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$.

Table 3 a) Condensed tannin concentration (g kg^{-1}DM), biological activity (g tannic acid equivalent/100gDM), mean degree of polymerisation (mDP), prodelphinidins (PD) (% in CT), c*is* flavanol content (% *cis* in CT) of condensed tannins (CT) of three sainfoin varieties at different dates during the first vegetation cycle.

First growth cycle													
	Harvest Date												
	30 April	5 May	12 May	19 May	26 May	2 June	9 June	16 June	23 June	s.e.	Var	Date	Var×Date
Concentration	3.66	3.96	5.08	4.85	6.40	6.02	6.02	5.77	5.72	0.150	***	***	NS
Biological activity	1.09	1.10	1.14	1.06	0.98	0.88	0.87	0.87	0.85	0.010	***	***	NS
mDP	26.9	25.0	25.0	24.2	33.3	36.4	2.2	45.5	47.7	0.46	***	***	*
Prodelphinidins	64.7	63.6	62.8	64.8	66.5	68.00	70.9	73.2	74.2	0.18	**	***	NS
Cis flavanol	73.0	72.6	72.7	75.2	79.6	81.4	82.2	83.1	82.0	0.490	***	***	NS

Variety (Var), s.e. standard error of means, NS, non significant, *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$.

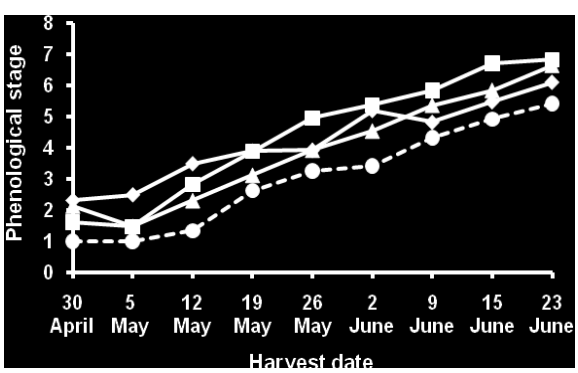
Table 3 b) Condensed tannin concentration (g kg^{-1}DM), biological activity (g tannic acid equivalent/100gDM), mean degree of polymerisation (mDP), prodelphinidins (PD) (% in CT), c*is* flavanol content (% *cis* in CT) of condensed tannins (CT) of three sainfoin varieties at different dates during the second vegetation cycle

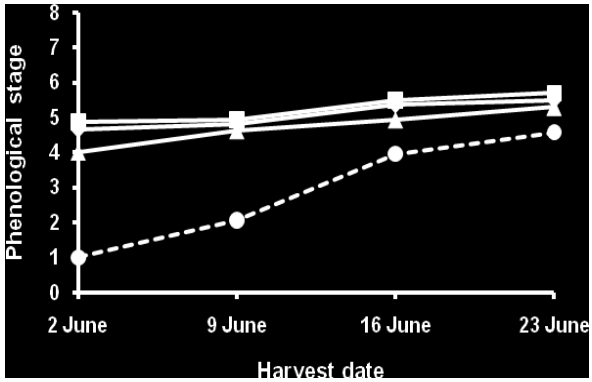
Second growth cycle								
	Harvest date							
	2 June	9 June	16 June	23 June	s.e.	Var	Date	Var*date
Concentration	6.98	7.8	8.46	8.04	0.344	***	NS	NS
Biological activity	1.42	1.36	1.34	1.12	0.03	***	***	NS
mDP	37.4	38.0	44.4	49.8	0.611	NS	***	NS
Prodelphinidins	69.6	65.3	71.7	70.5	0.333	NS	***	NS
Cis flavanol	75.3	77.5	81.5	81.0	0.670	***	***	NS

Variety (Var), s.e. standard error of mean, NS, not significant, *, $P < 0.05$; **, $P < 0.01$; *** $P < 0.001$.

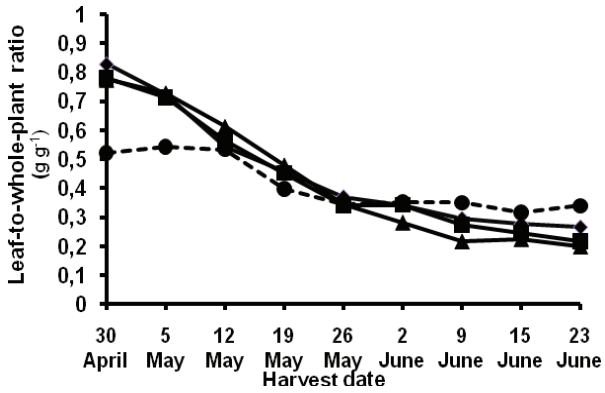
Figure 1 Evolution of phenological stage a) and leaf-to-whole-plant ratio b) at different harvest dates during the first and second growth cycles for three sainfoin varieties and a lucerne variety.

a) First growth cycle Second growth cycle





b)



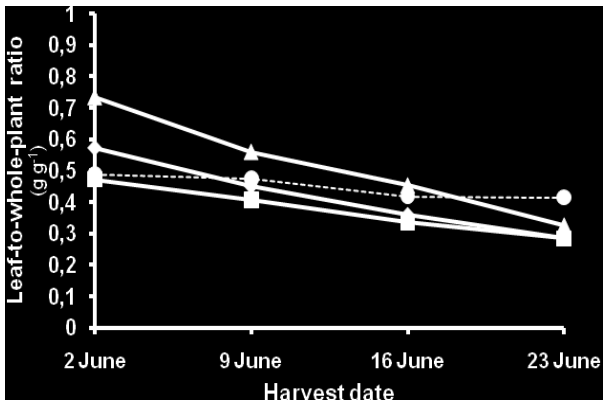
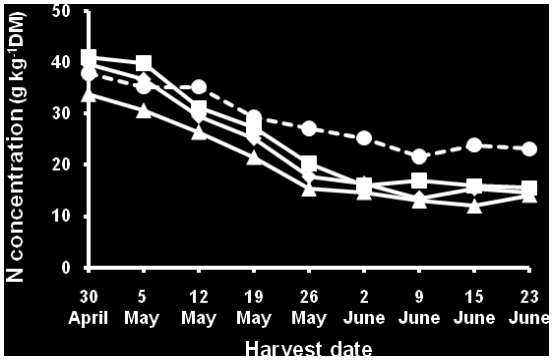
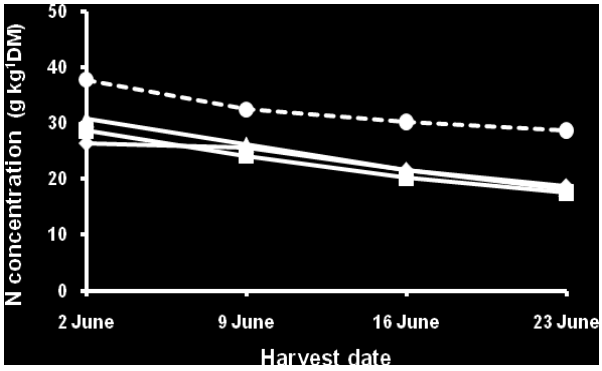


Figure 2 a) Nitrogen concentration of sainfoin varieties and lucerne and b) Nitrogen solubility with PEG and Nitrogen solubility without PEG) at different harvest dates during the first and second growth cycles.

a) First growth cycle Second growth cycle





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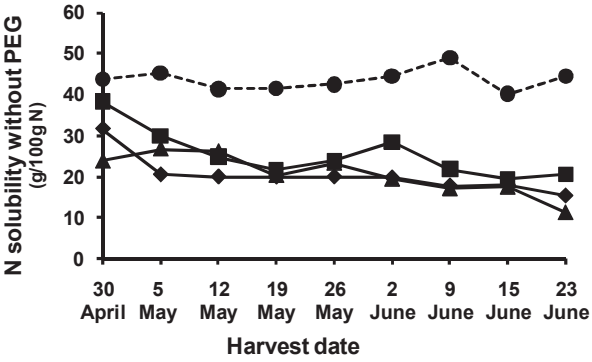
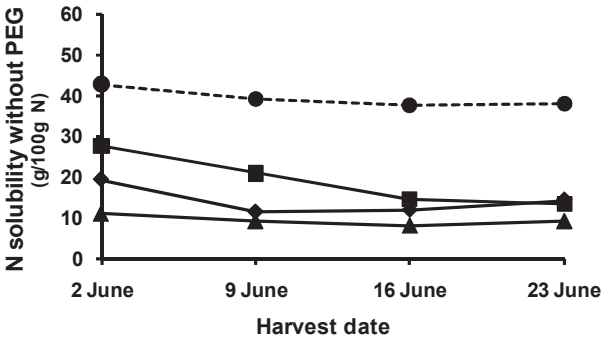
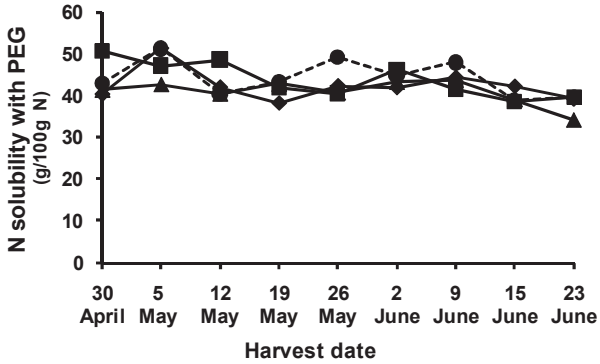




c)



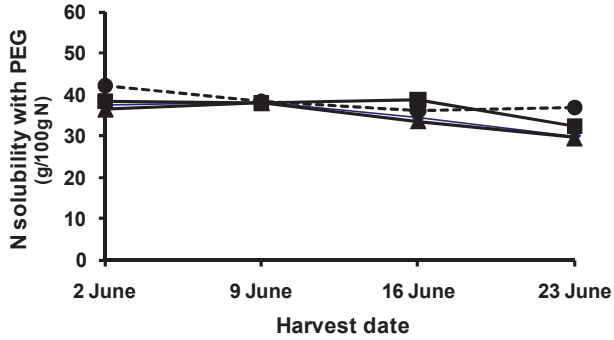
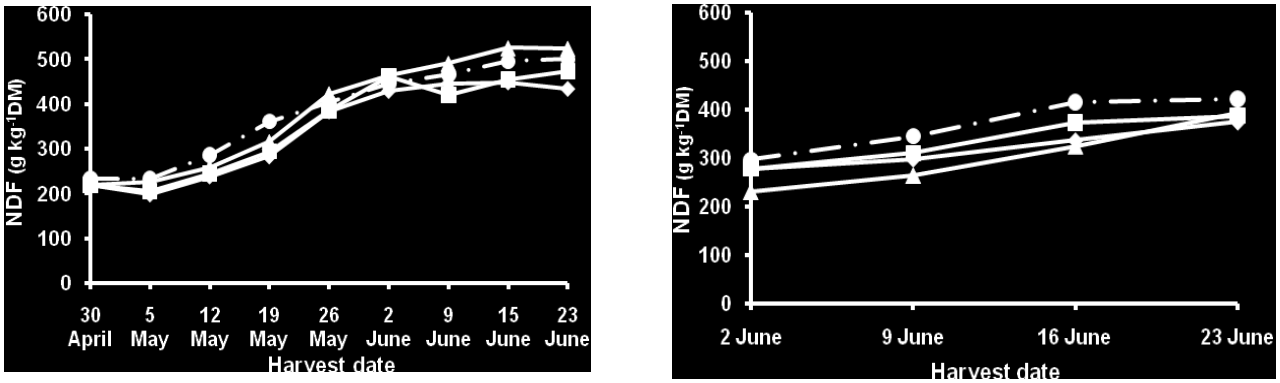


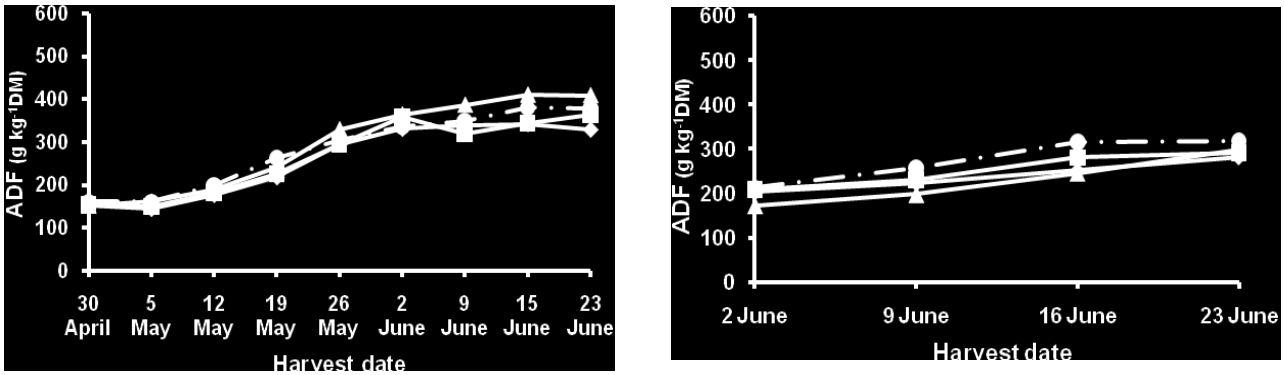
Figure 3 a) neutral detergent fiber (NDF), b) acid detergent fiber (ADF) and c) acid detergent lignin (ADL) at different harvest dates for three sainfoin varieties and a lucerne variety during the first and second growth cycles.

Ambra Villahoz Esparcette Lucerne

a) First growth cycle Second growth cycle



b)



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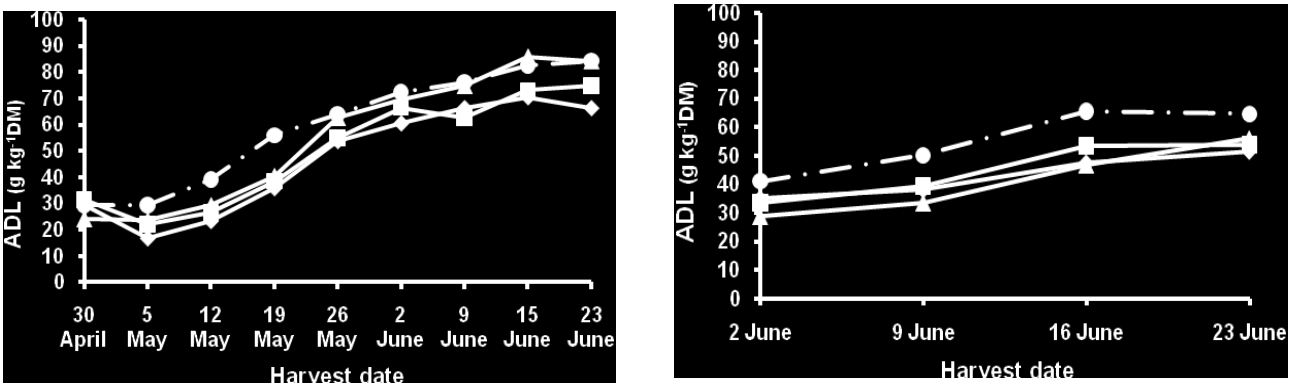


Figure 4 Pepsin-cellulase digestibility at different harvest dates for three sainfoin varieties and a lucerne variety during the first and second growth cycles. —◆— Ambra —■— Villahoz —▲— Esparcette - -●- - Lucerne

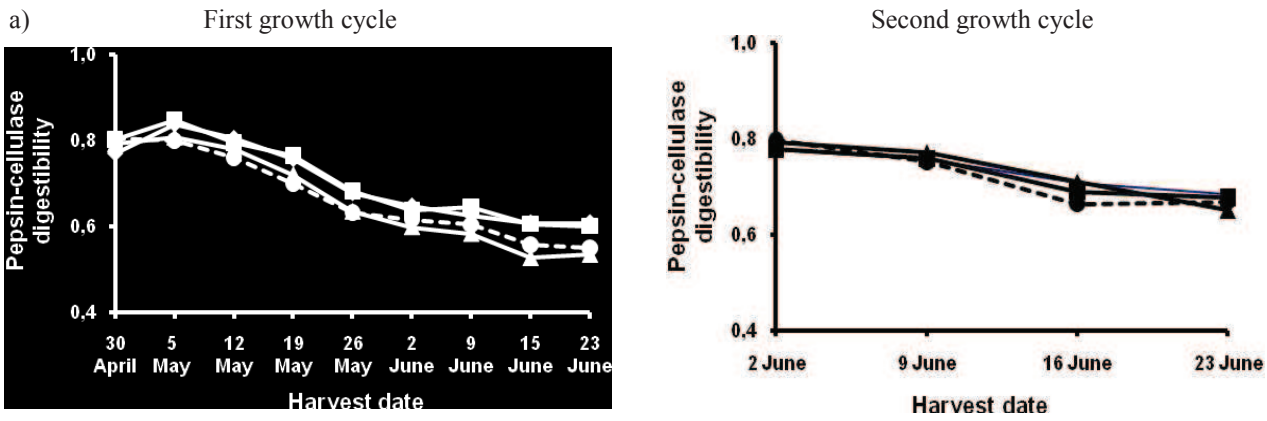
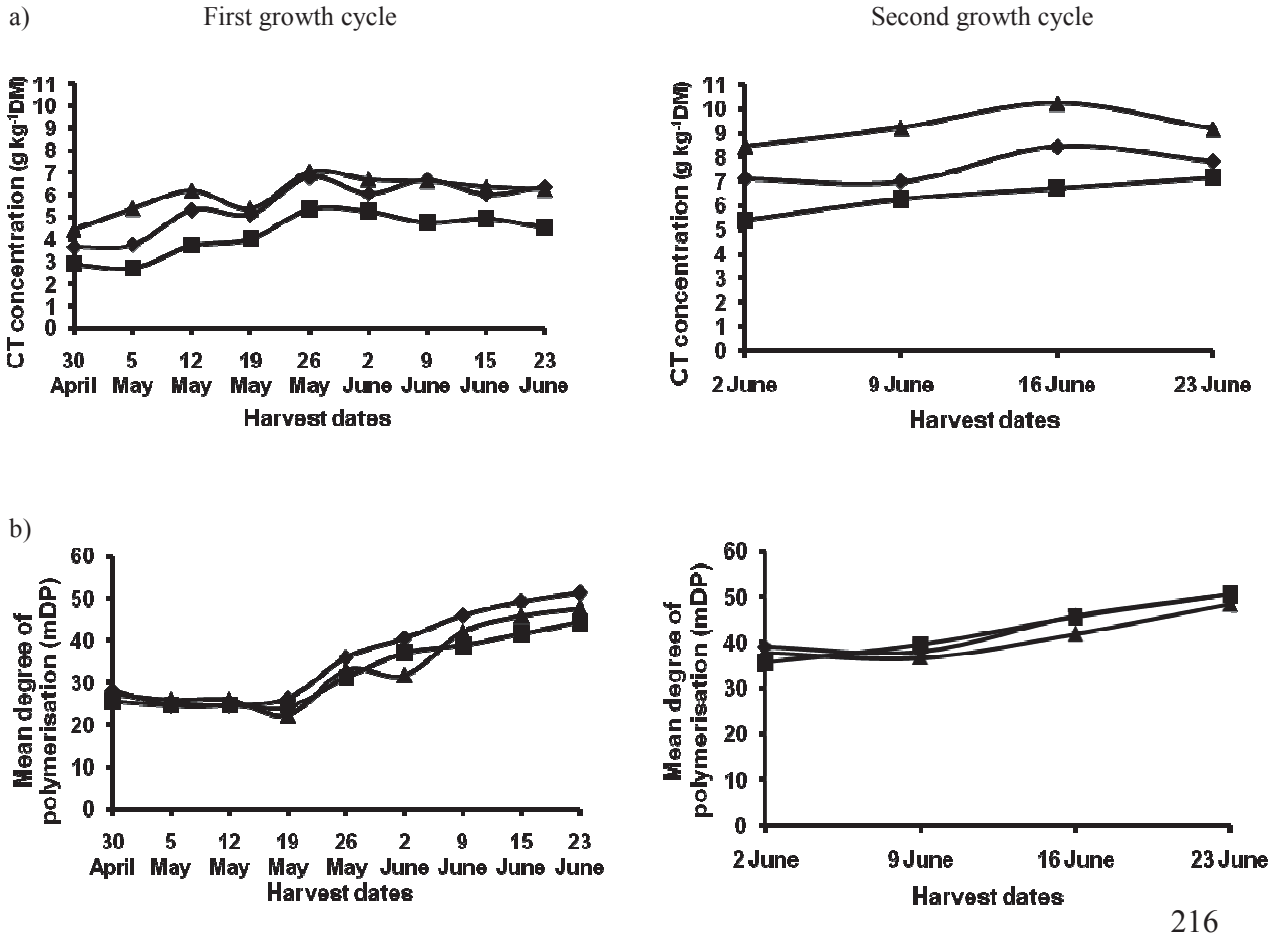
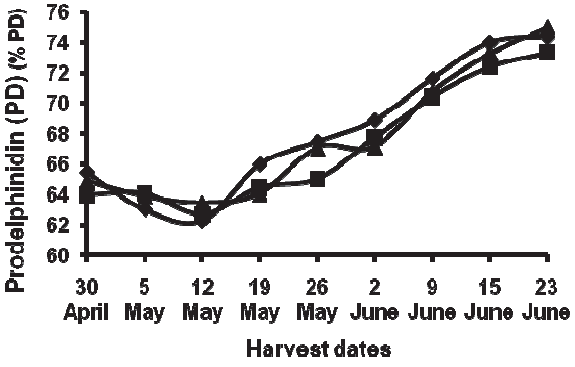
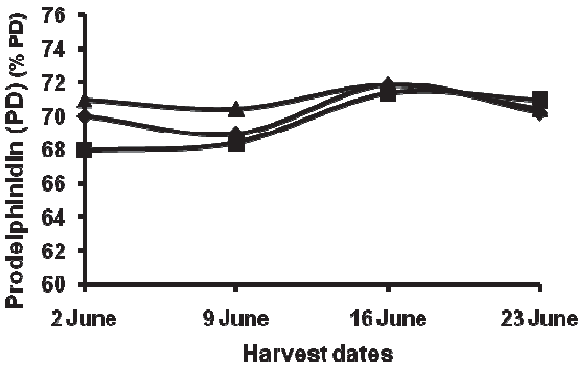


Figure 5 Condensed tannin (CT) parameters at different harvest dates for three sainfoin varieties during the first and second growth cycles. a) concentration (g kg^{-1}DM), b) mean degree of polymerisation (mDP), c) prodelphinidin content (% PD in CT), d) *cis* flavanol content (% *cis* in CT), e) biological activity (g tannic acid equivalent/100g DM). —◆— Ambra —■— Villahoz —▲— Esparcette - -●- - Lucerne

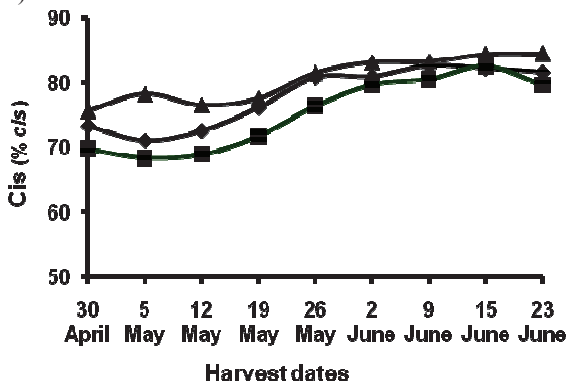


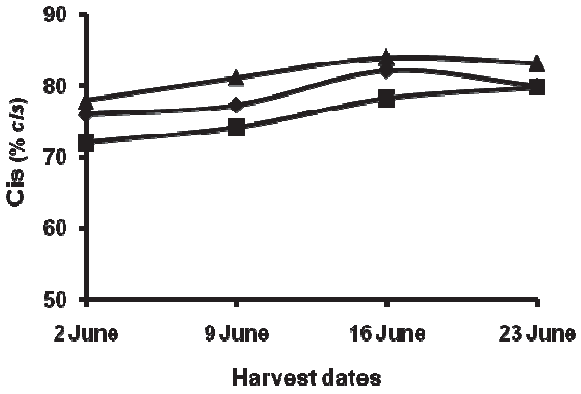
c)


Prodelphinidin (PD) (% PD)
76
74
72
70
68
66
64
62
60
30 April
5 May
12 May
19 May
26 May
2 June
9 June
15 June
23 June
Harvest dates

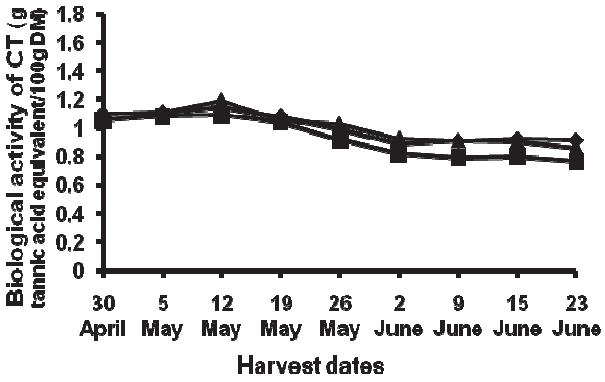

Prodelphinidin (PD) (% PD)
76
74
72
70
68
66
64
62
60
2 June
9 June
16 June
23 June
Harvest dates

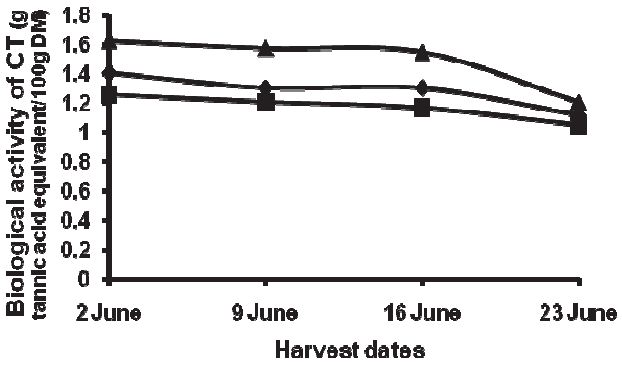
d)


Cis (% c/s)
90
80
70
60
50
30 April
5 May
12 May
19 May
26 May
2 June
9 June
15 June
23 June
Harvest dates


Cis (% c/s)
90
80
70
60
50
2 June
9 June
16 June
23 June
Harvest dates

e)


Biological activity of CT (g tannic acid equivalent/100g DM)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
30 April
5 May
12 May
19 May
26 May
2 June
9 June
15 June
23 June
Harvest dates


Biological activity of CT (g tannic acid equivalent/100g DM)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2 June
9 June
16 June
23 June
Harvest dates

Chapter 4
Discussion

General discussion

The objective of my thesis was to extend our knowledge of the nutritional value of the sainfoin forage legume and its environmental advantages when feeding to ruminants. In particular, the effects of condensed tannins (CT) were assessed for reducing nitrogen excretion and other gas emissions.

The experimental work we carried out enabled us to analyse the effects of CT in sainfoin on its digestion and nutritional value according to its phenological stage and preservation method, and relate them to the CT content and structures. Our general discussion will successively address thses three issues that we identified.

i. **How do the structures, contents and biological activity of condensed tannins (CT) in sainfoin change according to phenological stage, vegetation cycle, preservation method and the plant variety studied?**

In this first part relationships were assessed among different methods for measuring CT contents and structures of four sainfoin varieties (Ambra, Esparcette, Villahoz and Perly) and between plant parts (stems, leaves and whole plant) of the Perly variety. CT contents and composition in terms of tannin size (mDP), % PD, % *cis* contents were then related to the chemical composition of the sainfoin varieties. Lastly, the time course of CT characteristics is related to phenological stages and vegetation cycles (Experiments 1 and 4).

ii. **What are the effects of CT in sainfoin on the digestive processes that determine its nutritional value and the environmental impact of its digestive utilisation?**

In this second part, we discuss the results obtained *in vivo, in situ* and *in vitro* that assessed the role of CT on dry matter and nitrogen utilisation. In particular, we focussed on their implications for ruminant nutrition (organic matter digestibility and nitrogen retention) and for the environment (nitrogen excretion and ruminal methane (CH_4) emission) (Experiments 1, 2 and 3).

iii. **What quantitative assessment of the nutritional value of sainfoin and its digestive utilisation can we propose?**

In this third part, we combine the results and propose a quantitative assessment of the nutritional value of sainfoin in comparison with other tannin-containing and tannin-free forage legumes (Experiment 1, 3 and 4).

I. CONTENT, STRUCTURE AND BIOLOGICAL ACTIVITY OF CONDENSED TANNINS IN SAINFOIN

1. Methods used for quantifying CT

CT content was determined by thiolysis and HCl-butanol methods, while the biological activity was determined by the radial diffusion assay. It is also known that the thiolysis method includes some residual tannin that cannot be extracted with solvents. The thiolysis method, provides both quantitative data on CT content and qualitative data on CT structural parameters (i.e. mean degree of polymerisation, mDP; and prodelphinidin:procyanidin, PD:PC and *cis:trans rations*) that may influence their biological activity (Schofield *et al*., 2001). In addition, we used the HCl-butanol method to confirm the presence of CT in our sainfoin material. In our laboratory we also used the radial diffusion assay (RDA; Makkar et al., 2000) which allows the biological activity of CT to be determined in terms of protein-binding capacity. This assay is suitable for screening large numbers of samples, including those of unusual composition, and requires neither complex reagents nor instruments (Hagerman, 1987).
All the methods were applied to freeze-dried samples. CT values obtained by the thiolysis and HCl-butanol methods represent the extractable plus unextractable CT, whereas radial diffusion assay determined only soluble tannins from the solvent extract.

There was a close correlation (R^2=0.964) between the HCl-butanol results and the biological activity of CT (RDA assay) for whole plant samples among the sainfoin varieties, and leaves and stems of the Perly variety (**Figure 12**).

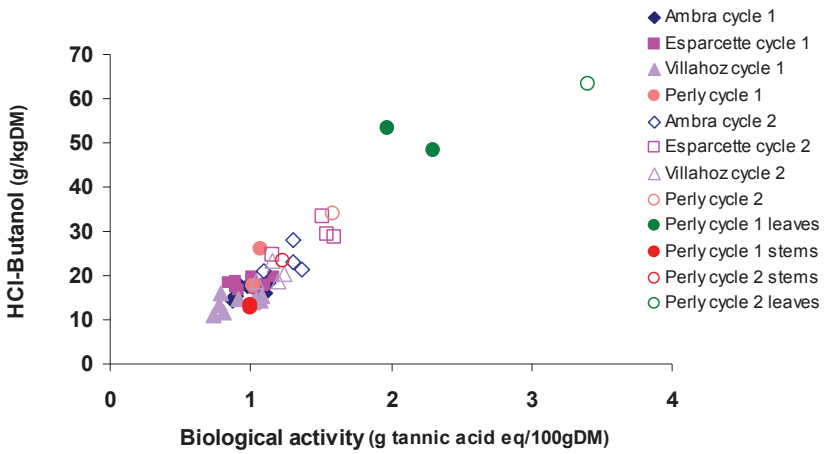


Figure 12: Relationship between HCl-butanol results and biological activity of CT for the whole plant of sainfoin varieties.

However, the correlation is hardly less close (R^2=0.899) when samples of leaves, which contain more CT, are not included in the regression.

The correlation between the thiolysis CT content and the biological activity of CT for all samples (except leaves of Perly variety) was slightly lower (R^2=0.771).

The relationship (R^2=0.660) between the HCl-butanol and thiolysis results (we did not include the samples of Perly cycle 2 variety) indicates that both methods are suitable for quantifying CT in different sainfoin varieties.

On the other hand, the value for the CT content of the whole plant of Perly variety at cycle 2 (13.6 g/kg DM) obtained by the thiolysis method, was quite high and differentiated this sample from the other sainfoin samples (**Figure 13**). For this reason, we recalculated its CT content by the percentage of leaves and stems, and the value obtained (9.4g/kg DM) was more realistic. However, the high residual standard deviation (RSD=0.69) obtained between the two methods (without the sample of Perly cycle 2) was partly the consequence of the low repeatability of the thiolysis method, the variability among the varieties, and possibly also the different solutions used for the extraction in the two methods.

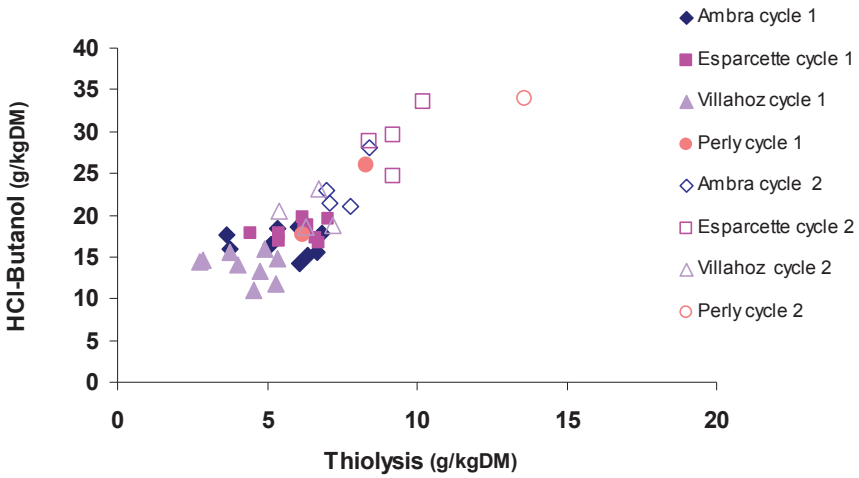


Figure 13: Relationship betweenCT contents in whole sainfoin plants measured by two different methods (HCl-butanol and thiolysis).

We could not compare the two analytical methods for analysing CT in wrapped sainfoin silage bales (WS), as the newly developed thiolysis method was apparently too mild (reaction occurs at 40^{0}C) and it could not detect CT in these WS samples. Schofield *et al*., 2001 previously reported that thiolysis conditions need to be optimised in order to achieve complete degradation. Matthews *et al*., 1997 studied bark tannins from *Picea sitchensis*, where the maximum thiolysis yield was 63%. However, Labarde *et al*., 1999

used more highly purified grape CT and the thiolysis yield was 88%. It is not clear to what extent structural differences or differences in reaction conditions contributed to these different outcomes. According to previous references, we can suppose that all these could provide possible reasons for the difficulties we faced in analysing our samples of wrapped silage sainfoin (WS). The higher CT content in the whole plant of the Perly variety than in leaves can also be explained by this hypothesis. The case of incomplete thiolysis may partly explain the less close correlation observed between thiolysis results and biological activity of CT, compared with the correlation between HCl-butanol results and biological activity.

In addition, alterations in the CT partition occur during the ensilage procedure. Cell rupture and release of the CT from the plant cells were caused by the ensiling process, allowing them to bind to other molecules. The amount of 'free' CT (CT not in complexes with other molecules) is markedly reduced in ensiled forages (Minnee *et al*., 2002; Lorenz *et al*., 2010). The remaining CTs are 'bound' to other molecules (protein or fibre). This may influence the reliability and effectiveness of the thiolysis method and make it unsuitable for CT analysis of silage samples. For all the above reasons, the results for condensed tannins in wrapped big bales of sainfoin should be viewed with caution.

2. The structure of CT and its relationship with biological activity

The literature contains little information on the relationships between CT structures and the biological activity of sainfoin CT.

From the figure below, it can be seen that several **parameters that characterize the structure of CT are correlated.** There was a close correlation (Perly cycle 2 was not included) (R^2=0.832, RSD=1.50) between the proportion in prodelphinidin units (PD) for whole sainfoin plants and the mDP, as well as between PD values and *cis* or mDP (R^2=0.748 and 0.867, respectively), consistent with the results of De Freitas and Mateus 2001 (**Figure 14**).

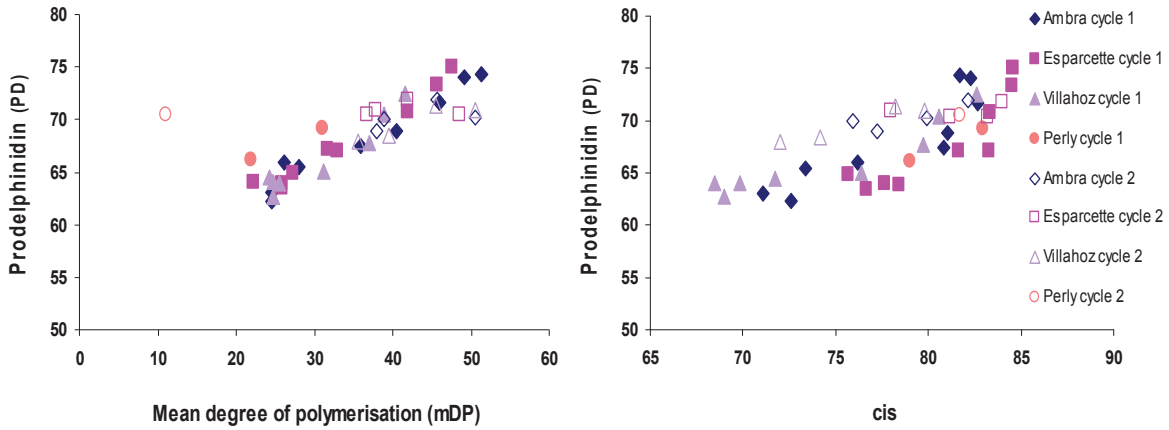


Figure 14: Relationship between prodelphinidin (% PD) and mean degree of polymerisation (mDP) or *cis* of whole sainfoin plants.

The mDP values can vary widely with environmental factors and sainfoin variety. The environmental conditions can result in changes in the relative expression of key enzymes in the biosynthetic pathway of CT, which can effectively alter the structure of the resulting product (Morris and Robbins, 1997). The CT concentration can vary with light intensity (Mole *et al*. 1988), fertilization (Lascano *et al*., 2001), temperature and plant maturity. Environmental factors can impact on CT concentration abd also affect CT structures (Barahona, 1999).

All of these factors could explain the differences that were found between the values measured in Experiment 1 and in Experiment 4 i.e. different cultivation years, altitudes (320 m *vs*. 870 m.), soils (fertile loamy soil *vs.* granitic brown soil) and sainfoin varieties (Perly *vs.* Esparcette, Ambra, and Villahoz).

As stated above the effect of variety is quite marked. The near infrared spectroscopy (NIR) spectra used in the laboratory to compare sainfoin varieties (Ambra, Esparcette, Villahoz, Perly), indicated that the variety Perly did not belong to the same population (distance $H>3$) as the other sainfoin varieties. However within our results, it is not possible to separate the environmental effects from the effect of variety (Perly), as Perly was cultivated in another environment. The results obtained from the 40 varieties, used in the European "Healthy Hay" network, grown in the same area, indicated a wide variation of the mDP values from 12 to 74. Hence the CT characteristics are greatly influenced not only by environmental factors, but also by the genetic variation among the varieties or cultivars.

The relationship between CT parameters (mDP, PD and *cis*) and biological activity differed between the two vegetation cycles (**Figure 15**). For a given mDP value, the biological activity was higher for the second than for the first vegetation cycle. The

biological activity decreased as the mDP increased (R^2=0.652 and 0.478) for the first and the second vegetation cycles, respectively; Perly cycle 2 not included).

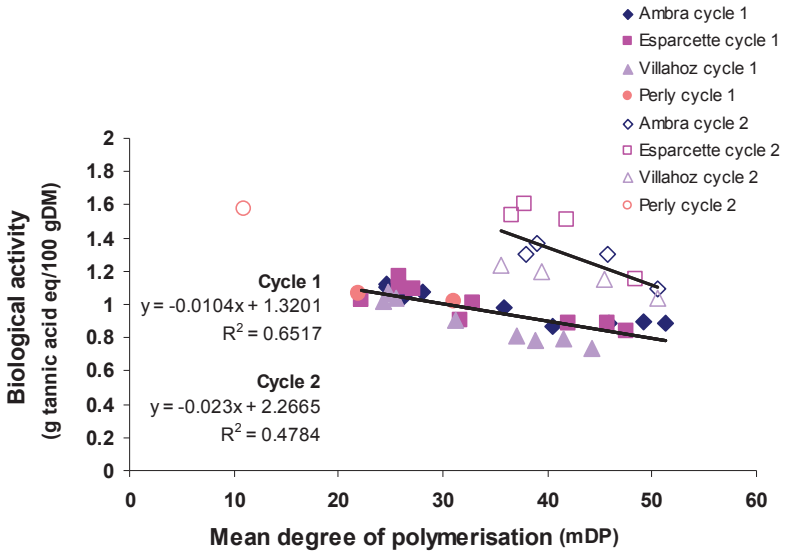


Figure 15: Relationship between biological activity of CT and mDP for sainfoin varieties.

This is in line with studies, which shown that the protein precipitation capacity (biological activity) was related to the molecular size of tannins (Frazier *et al*., 2010). Similarly, Ozawa *et al*. (1987) and Makkar *et al*. (1988) found that the protein precipitation capacity decreased with plant maturity, which was associated with an increase in the mean degree of polymerisation. This suggests that as the plant matured, the active sites on tannins for reaction with proteins became fewer in number or that tannin molecules became too large to interact effectively with the protein (Makkar *et al*., 1988). The proportion of PD units was also negatively related to biological activity at the first vegetation cycle (R^2=0.661) but there was no significant relationship at the second cycle. On the other hand, these results (Experiment 1 and Experiment 4) do not completely agree with some references in the literature that recognised that the biological activity of 'astringency' of CT of grape seed tannins is positively related to the mDP (Sarni-Marchado *et al*., 1999; Frazier *et al*., 2010). Huang *et al*. (2010) suggests that molecular mass of CT, at least for *leucaena* forages, is not the sole factor determining the capacity to bind protein. Also, for tanniniferous plants, tannin astringency also increases with PD content (Jones *et al.* 1976; Aerts *et al*., 1999).

Surprisingly, there was no significant relationship between CT content determined by the thiolysis or the HCl-butanol method and CT structure (mDP or PD parameters). In contrast, a close relationship was found between CT content (thiolysis method) and % *cis*

(R^2= 0.714 at the first and 0.820 at the second vegetation cycle with RSD = 0.67 and 0.62, respectively, excluding Perly cycle 2).

3. Relationship between CT properties and morphological or chemical composition of sainfoin

The biological activity of CT increased with the leaf-to-whole-plant ratio. For a given leaf-to-whole-plant ratio biological activity was higher for the second than for the first vegetation cycle. As a result, negative correlations were observed (R^2=0.670 and 0.412 for the first and second vegetation cycle, respectively, n=42) between biological activity of CT and NDF, and positive correlations between biological activity and N concentration or pepsin-cellulase digestibility (Dcell). However, for both vegetation cycles there was no significant correlation between NDF and CT (**Figure 16**).

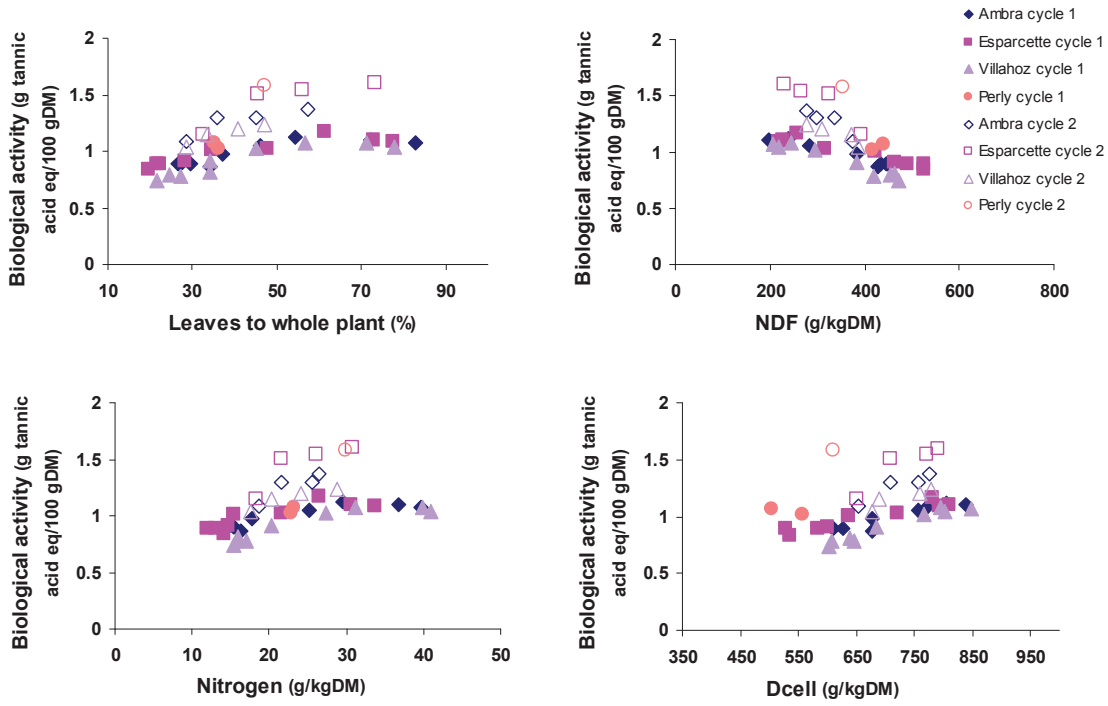


Figure 16: Relationships between biological activity of CT and leaves-to-whole-plant ratio, NDF, nitrogen and Dcell for Perly and the three varieties (Ambra, Villahoz, Esparcette).

A negative relationship was observed between the nitrogen solubility and CT content or biological activity, as well as with the different CT parameters such as % PD content (**Figure 17**). This revealed that effects on protein soloubility are proportional to CT content and activity. The relationship with CT parameters may be due to the fact that the

CT with higher PD and mDP values precipitated more proteins (Aerts *et al*., 1999), which resulted in lower N solubility.

For the Perly variety (stems, leaves and whole plant), n=9, the correlation between nitrogen solubility and HCl-butanol results or biological activity was very close (R^2=0.968 and 0.932, respectively). A close correlation was also observed between nitrogen solubility and % PD content of leaves, stems or whole plant of the Perly variety. Consistent with the literature, the presence of more CT in the leaves resulted in a lower nitrogen solubility.

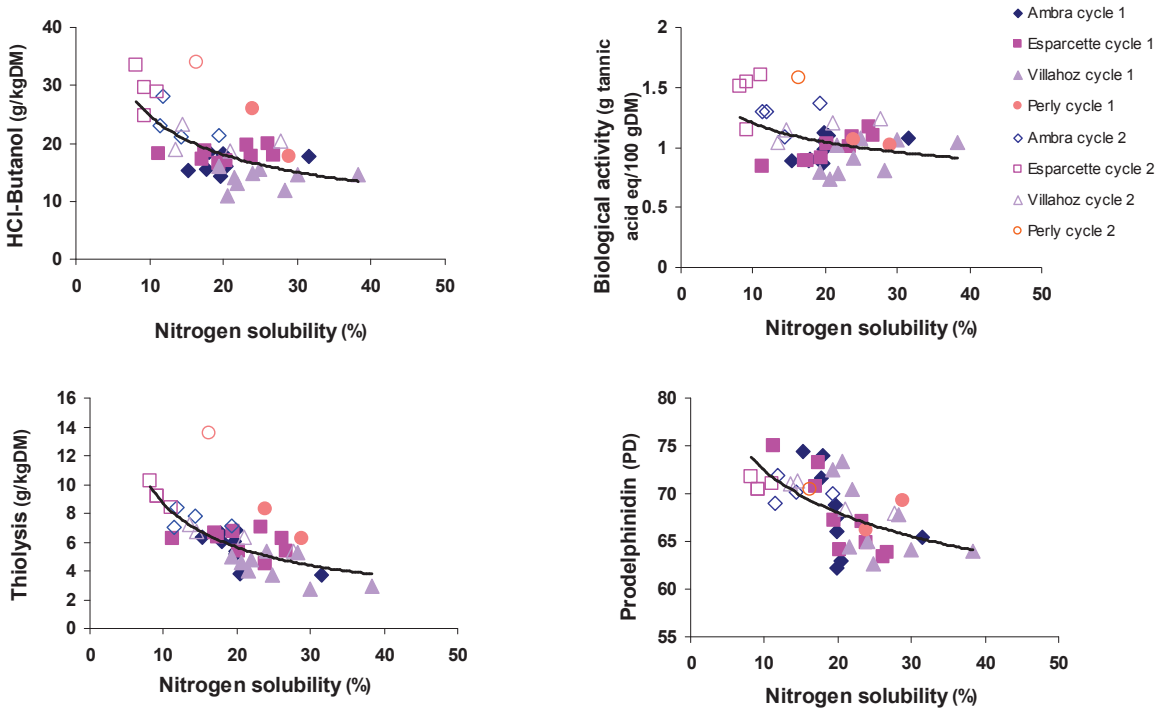


Figure 17: Relationships between CT content (HCL-butanol or thiolysis method), biological activity, prodelphinidin and nitrogen solubility of whole sainfoin plants.

4. Change in CT content, biological activity and structure with phenological stage

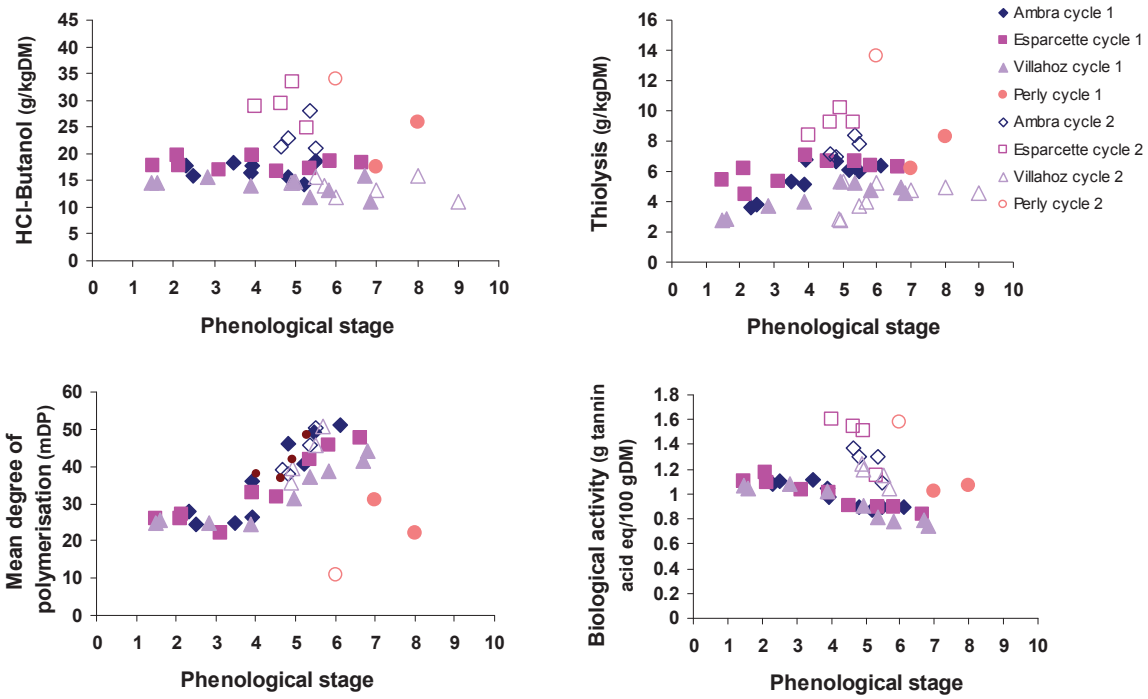


Figure 18: Relationship between CT content (HCL-butanol or thiolysis method), mean degree of polymerisation, biological activity and phenological stage of sainfoin varieties. The codes and definitions of the phenological stages in our study were as follows: 1=early vegetative, 2=mid-vegetative, 3=late vegetative, 4=late vegetative, 5=early bud, 6= late bud, 7=early flower, 8=late flower 9= early seed pod (Boreani *et al*., 2003).

For a given phenological stage, CT content was higher in the second than the first vegetation cycle, (n=42), regardless of the analytical method (thiolysis or HCl-butanol) (**Figure 18**). Higher CT contents were found at the second vegetation cycle even when compared for the same leaf-to-whole-plant ratio or for the same chemical composition. It is likely that the longer photoperiod and the higher temperature during the second vegetation cycle led to an increaced biosynthesis of tannins, as observed by Lees *et al*. (1994) on *Lotus*.

An unexpected result was that the CT content in Experiment 4 increased with the maturity of the plant: according to the literature, a lower leaf/whole plant ratio resulted in a lower CT content (Lees *et al*., 1995). The increase in CT content from the end of vegetative phase may be explained by the presence of CT-rich flowers (Gebrehiwot *et al*., 2002). Moreover, during the growth period, when plants produce mostly vegetative biomass, few resources are available for synthesis of phenolic compounds (Iason *et al*.,

1993). Despite the increase in CT content, the decrease in biological activity with phenological stage could be explained by a decrease of the proportion of extractable CT in the total CT of the plant. Tannins with higher molecular weights are more difficult to extract (Gea *et al*., 2011).

In Experiment 4, the mDP values increased with the phenological stage and were related with the decrease in biological activity. It is assumed that molecular size affects the ability of CT to form insoluble complexes with other molecules (Kumar and Vaithiyanathan, 1990). By contrast, in Experiment 1, mDP decreased for the mature fresh forage at the green seeds stage. This may be related to the process of fruit ripening described by Goldstein and Swain, 1963.

The correlation between % PD and the phenological stages were greater for the first vegetation cycle. In Experiment 1, %PD of the whole plants hardly varied among the three different phenological stages (66.2 to 70.5). The values we obtained were similar to those reported by Foo *et al*.,1982 while Marais *et al*., 2000 found a range from 36/64 to 93/7 on different sainfoin varieties. The PD content of 40 sainfoin varieties analysed in the Healthy Hay network ranged from 53 to 95. In Experiment 4, % PD was higher at the second vegetation cycle and increased with the phenological stage. The mean value of % PD in Experiment 1 was in the same range as in Experiment 4. However, both experiments were conducted under different environmental conditions, so comparing the results of Experiment 1 and 4 is somewhat difficult.

The % *cis* content increased with the phenological stage for the sainfoin varieties in Experiment 4. However, it did not vary between the vegetation cycles and among stems, leaves and whole plants in Experiment 1. The *cis:trans* stereochemistry of tannins may affect the three-dimensional structure of CT and their interaction with proteins (De Freitas and Mateus 2001), but there is little information on this in the literature.

Finally, as CT complex with proteins, we analysed, the change of N solubility in relation to phenological stage. Over all samples (n=44), N-solubility decreased only slightly with the phenological stage in the first and second vegetation cycle (**Figure 19**). This agreed with previous results (Aufrère *et al.*, 2005). This change stems from two opposing effects, which partially compensate each other: i) total N content, soluble N content and biological activity decreased with the phenological stage, and ii) lower biological activity tends to increase the N-solubility. It is note that N-solubility was higher for the wrapped silage bales and there was no growth stage. Ensiling appeared to have a beneficial effect on sainfoin N.

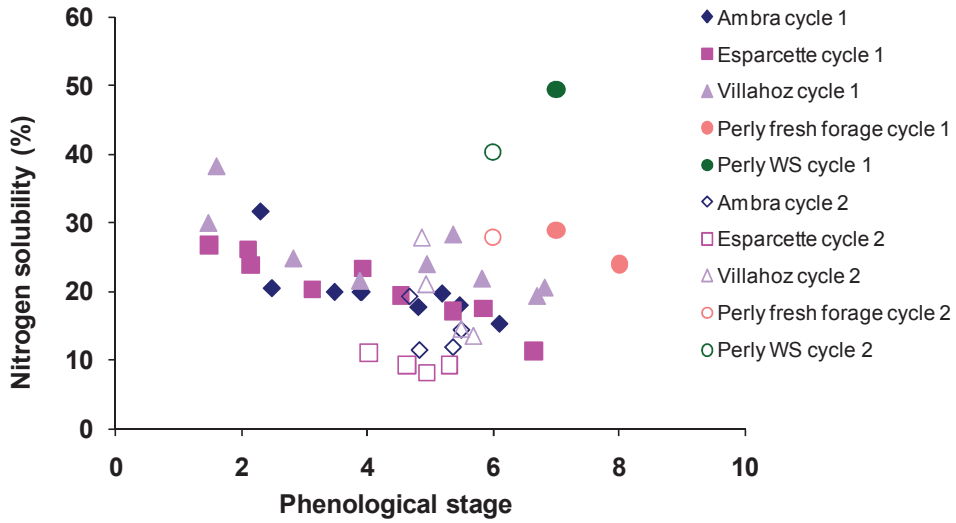


Figure 19: N-solubility at different phenological stages during the first vegetation cycle for fresh forages Perly, Ambra, Villahoz and Esparcette and wrapped silage bales (WS).

Conclusion

Our results show a close correlation between the CT content determined by the two methods (HCl butanol and thiolysis), between CT content and biological activity, and between biological activity and mDP, with an effect of the vegetation cycle. The main parameters of CT structure (mDP and PD) were also closely correlated. The differences among the varieties and vegetation cycle can partly be explained by differences in CT content and structure. The biological activity increases with leaf-to-whole-plant ratio, N content and Dcell, and decreases with NDF content. N-solubility decreases as % PD and CT content increase. Surprisingly, the CT content tended to increase slightly with the phenological stage, but the biological activity decreases with the increases with mDP, PD and *cis* values. Interestingly, the Perly variety was different from the other three varieties, but we cannot assert that this is a true variety effect, as Perly was cultivated in different environmental conditions- and this may warrant further study in the context of future plant breeding programmes.

II. EFFECTS OF CONDENSED TANNINS ON DIGESTIVE UTILISATION OF SAINFOIN

The effect of condensed tannins (CT) on digestive parameters was measured:

- *in vivo* using the whole plant and *in situ* in the rumen and the intestine using leaves and stems at three phenological stages (Experiment 1)
- *in vitro* on fresh forage (whole plant, leaves and stems) at two phenological stages (Experiment 2)
- and *in vivo* on wrapped silage bales of sainfoin (whole plant at two phenological stages) (Experiment 3).

The discussion focuses on two phenological stages: start of flowering and end of flowering, as they were common to all three experiments.

Polyethylene glycol (PEG) was used to demonstrate whether any of the observed effects were due to tannins or not. The water-soluble PEG not absorbed in the digestive tract can be used in numerous *in vivo* and *in vitro* studies to demonstrate the effects of tannins, as we indicated in the bibliographic section. As the CT had a greater affinity for PEG than for proteins, CT bind to PEG and the proteins are released. PEG contains sufficient oxygen molecules in a chain to form strong hydrogen bonds with the phenolic and hydroxyl groups in CT (Silanikove *et al*., 2001). However, the interpretation of the results is sometimes complex because PEG can also bind to other molecules (Palmer and Jones, 2000).

In addition, in our study, we performed an assay to examine the presence of polyphenol oxidase (PPO) in sainfoin. This enzyme, present in red clover (Jones *et al*., 1995) catalyses the oxidation of monophenols to diphenols and the oxidation of diphenols to produce *o*-quinones, which can react with proteins with covalent irreversible bonding. However, in our assay, no PPO activity was found in sainfoin, which means that all the results obtained were due to the presence of CT.

The overall effects of CT are being consider on digestive utilisation and nitrogen and gaseous losses and in relation to CT content, structure and biological activity.

1. Organic matter digestibility and dry matter disappearance (DisDM) in the rumen

Results from previous *in vivo* and *in vitro* studies have highlighted variable responses on organic matter digestibility due to plant. In our *in vivo* study, which fed sainfoin either as fresh forage (Experiment 1) or as wrapped silage (WS) (Experiment 3), organic matter digestibility (OMD) was significantly affected by CT only at the end of flowering (although values were slightly lower in the presence of active tannins in both vegetation cycles).

No significant effect of CT was observed for the whole plant in our *in vitro* true organic matter digestibility (IVTOMD) (Experiment 2). No effect was observed on IVTOMD of leaves and stems at 3.5 h, whereas at 24 h of incubation, CT increased IVTOMD despite a decrease in CH_4 production (**Table 5**). However, the decrease in IVTOMD observed in Experiment 2 by PEG addition may also be due to an artefact of insoluble complexes, which can be formed with other forage components, such as fibre, which remains in the residue, results in an underestimation of IVTOMD (Palmer and Jones, 2000; Makkar, 2000). As expected, for both fresh forages and WS, OMD was higher at the start of flowering than at the end of flowering.

Table 5: Effect of condensed tannins on *in vivo* organic matter digestibility (OMD) and *in vitro* true organic matter digestibility (IVTOMD).

Phenological stage	End of flowering		Start of flowering	
PEG treatment	with	without	with	without
***In vivo* OMD**				
Fresh forage	581	607	648	654
Wrapped silage bales	567	541	627	624
***In vitro* IVTOMD**				
3.5 h of incubation				
Whole plant	501	505	608	610
Leaves	646	650	649	656
Stems	449	451	572	580
24 h of incubation				
Whole plant	678	680	747	767
Leaves	783	807	772	828
Stems	626	629	733	751

DisDM in the rumen (**Table 6**) was measured *in situ* using the nylon bag procedure. CT decreased ruminal DisDM of leaves and stems, which was unexpected, as the organic matter digestibility (OMD) was not affected by the presence of CT. Various hypotheses may be considered for this observation. DM disappearance in the rumen could be effectively reduced in the presence of active CT, and compensatory degradation in the large intestine may explain the absence of effect on the total tract OM digestibility. However, as it is known that PEG addition increases the transit rate from the rumen (Schiller *et al*., 1988), it is not justified to compare the values of DisDM with and without PEG using the same value of passage rate ("kp" = 0.06/h) for the calculation of DisDM. Thus to take into account the effect of PEG on passage rate we recalculate the values of DisDM without PEG with a lower value of "kp" equal to 0.05/h (**Table 6**). From these calculations it can be seen that the differences of DisDM with and without PEG are lowered or eliminated by considering that PEG increases passage rate by 0.01/h, which seems realistic.

Table 6: Calculations of the DisDM with a "kp"=0.05/h for Perly variety without PEG addition at three phenological stages.

Phenological stage	End of flowering	Green seeds	Start of flowering
Stems without PEG		**DisDM**	
kp 0.05 /h	0.359	0.341	0.489
kp 0.06/h	0.326	0.313	0.457
Stems with PEG			
kp 0.06/h	0.377	0.332	0.477
Leaves without PEG		**DisDM**	
kp 0.05/h	0.538	0.534	0.546
kp 0.06/h	0.503	0.491	0.507
Leaves with PEG			
kp 0.06/h	0.616	0.539	0.577

2. Nitrogen digestion

The nitrogen digestibility for the whole animal takes a global approach to nitrogen metabolism in the digestive tract. Nitrogen balance studies enables to measure the quantity of nitrogen retained by ruminants and to calculate the proportion of nitrogen excreted in faeces and urine as a function of the nitrogen intake.

The digestive processes can be analysed from measurements made in the different compartments of the digestive tract (kinetics of the tN and NH_3-N content in the rumen fluid and measurements of protein degradation in the rumen by the nylon bag method), and disappearance of nitrogen in the intestine using the mobile bag technique.

N retention and total tract N digestibility

The difference between total tract N digestibility measured with or without PEG addition was more noticeable for the less mature, fresh forage of Perly variety (**Table 7**). This could be due to the higher CT content and biological activity or the lower mDP values at the start of flowering (n=4) (**Figure 20**). The same results at the start of flowering were observed for the WS, and in that case, this can be interpreted only with the higher CT content and biological activity as no data on CT structure were available.

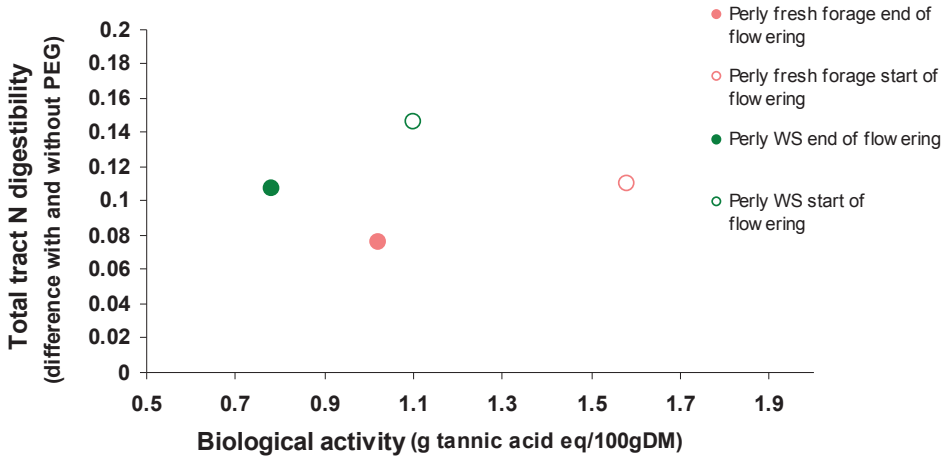


Figure 20: Difference of total tract N digestibility with and without PEG and the biological activity of the whole plant of fresh and wrapped silage bales of sainfoin Perly.

For ruminant nutrition, it is important that during the Experiment 1 and 3, despite the decrease in N digestibility by CT, that the overall N retention was not affected. For fresh forage and wrapped silage bales N retention was also higher for the less mature forage, as a result of higher N intake and digestibility.

Table 7: Total tract organic matter and N digestibility, N losses and N retention for sheep fed fresh and wrapped silages bales of sainfoin at the end of flowering and start of flowering with and without PEG addition in the rumen.

	Fresh forage				Wrapped silage bales			
Phenological stage	End of flowering		Start of flowering		End of flowering		Start of flowering	
PEG treatment	with	without	with	without	with	without	with	without
OM	581	607	648	654	567	541	627	624
N (total tract)	677	601	775	665	655	548	737	591
N (g/g N intake)								
Faeces	0.319	0.399	0.224	0.336	0.346	0.442	0.261	0.408
Urine	0.454	0.410	0.478	0.370	0.417	0.400	0.455	0.321
Nitrogen retained								
g/d/ sheep	6.77	5.49	12.72	12.30	7.66	4.93	12.12	11.22
g/g N intake	0.23	0.19	0.30	0.29	0.23	0.16	0.28	0.27

Nitrogen disappearance (DisN) in the rumen

The DisN in the rumen were measured *in situ* using the nylon bag procedure. Despite the higher N content of leaves compared with stems, the DisN of leaves was lower than stems due to the higher CT content (measured by HCl-butanol) and biological activity in leaves. The same results on the same forages were observed in our *in vitro* study (n=6) (Experiment 2) (**Figure 21**). The differences between leaves and stems DisN could also be partly explained by the greater indigestible N concentrations in leaves than in stems. Leaves, because of a greater amount of primary cell walls, would be expected to have greater concentration of cell-wall N than stems, which have more secondary cell walls (Sanderson and Wedin, 1989).

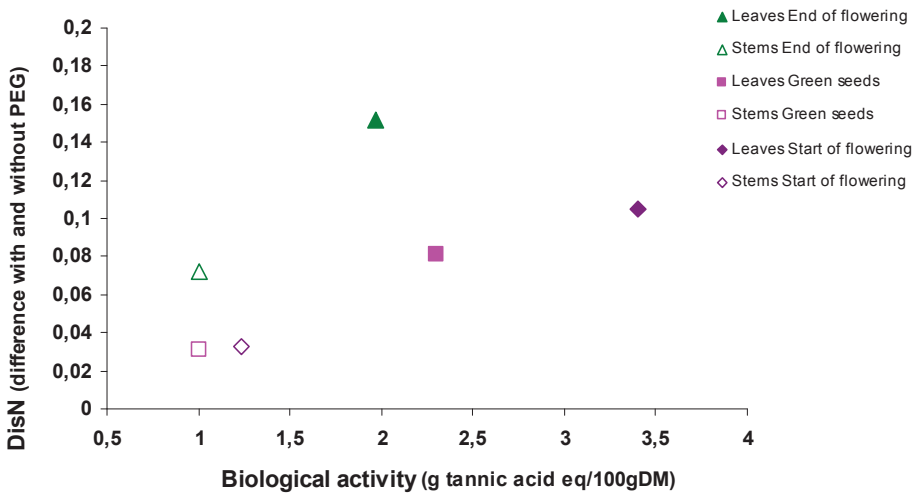


Figure 21: Relationship between nitrogen disappearance in the rumen (DisN) and biological activity of CT.

The smaller PEG effect with stems was related to their lower CT content (measured by HCl-butanol) and biological activity. However, the less marked CT effect on DisN for leaves and stems at the start of flowering compared with the end of flowering was unexpected. This may be due to insufficient quantity of PEG so that not all tannins wew bound at this phenological stage.

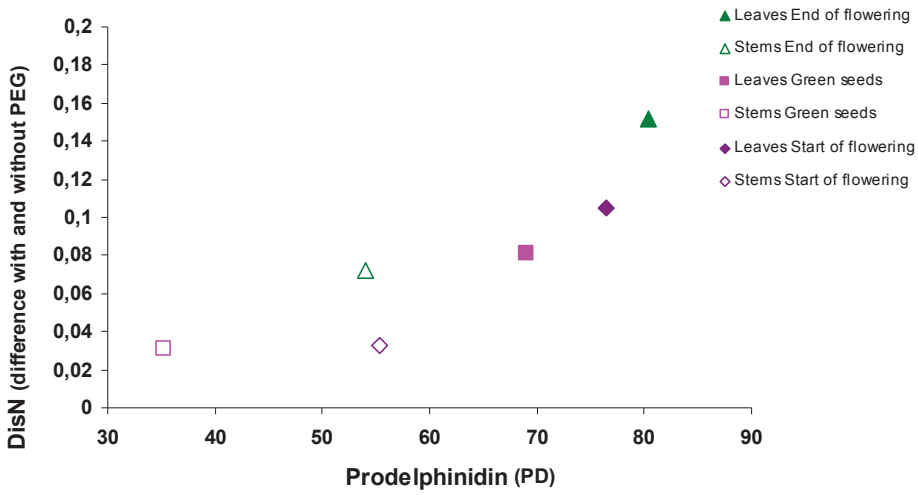


Figure 22: Relationship between nitrogen disappearance in the rumen (DisN) and prodelphinidins (PD) unit of CT.

It is noteworthy that the differences between DisN values obtained with and without PEG for stems and leaves were closely related with the proportion of PD unit in CT (R^2=0.766). As presented in **Figure 22**, higher PD content resulted in higher DisN differences.

Total nitrogen (tN) and ammonia nitrogen content (NH3-N) in the rumen fluid

The tN and NH_3-N contents measured in rumen fluid reflect the rate of degradation of rapidly degradable nitrogeneous fractions. These measurements were determined on the Perly variety, *in vivo* as fresh forage (Experiment 1), preserved as wrapped bales (Experiment 3) and *in vitro* on fresh forages.

In Experiment 1, whatever the vegetation cycle and the phenological stage of sainfoin, tN and NH_3-N in the rumen fluid peaked 1.5 h after feeding and then declined up to 6 h after feeding. Ct decreased the mean tN and NH_3-N content in the rumen fluid and this effect was higher for the second vegetation cycle. In accordance with the decrease in tN and NH3-N, the disappearance rate of fraction 'b' which was measured *in situ* in the rumen by the nylon bag method, was also decreased by CT. Surprisingly, no effect of CT on tN and NH3-N was observed with the WS. As stated in Part I, a decrease in the amount of "free CT " and an increase of fibre-bound CT during ensiling resulted in less CT available for binding to PEG.

Data obtained for NH3-N in *in vitro* measurements (Experiment 2) agree very well with the *in vivo* data. The presence of sainfoin CT resulted in lower NH3-N content at both incubation times (3.5 h and 24 h) for leaves, stems and the whole plant. The difference between NH_3-N productions measured with and without PEG, for all parts of the plants at 3.5 h and 24 h of incubation, was markedly higher at the start of flowering and was greater for leaves than for stems. This is consistent with the higher N, CT content and biological activity in leaves than in stems in a less mature plant.

***In situ* nitrogen digestion in the small intestine**

The lower N disappearance in the rumen can only be considered as a beneficial effect of CT if it is associated with an increased absorption of amino acids in the small intestine (Aerts *et al*., 1999; Barry and McNabb, 1999). In our study (Experiment 1) the reduced rumen degradation of leaf protein by CT was balanced by an increase in the amount of N digested in the small intestine as the amount of N entering the intestine was enhanced. On the other hand, CT did not affect N disappearance of stems in the small intestine. The distinct structural differences between leaf and stem CT (i.e. PD mDP, *cis* parameters) as well as their lower CT content and biological activity may explain the absence of a CT effect on stem N disappearing from the small intestine. Finally, as the leaves contain most of the digestible forage N, the presence of CT in sainfoin did not reduce the amount of N that could be digested by the ruminant. This was confirmed by the amount of N retained which was not reduced (**Table 7**) by active tannins for the less mature stage (start of flowering).

3. N excretion and methane emissions

Tanniniferous feeds can alter the form of excreted N and reduce environmental N pollution from ruminants. Lower protein degradability tends to reduce ruminal NH3 production and may thus lead to lower urinary N excretion (Min *et al*., 2003b). Plants tannins are also of interest for reducing global methane emission (Jouany and Morgavi, 2007), which is the main greenhouse gas (GHG) produced by ruminants. The efficacy of sainfoin for these purposes is not well documented in the literature.

Nitrogen excretion

For fresh and preserved forages (Experiments 1 and 3), active CT compared with PEG inactive CT decreased urinary N and increased faecal N excretion. This shift of nitrogen partition is beneficial to the environment for several reasons. Firstly, it is known that ammonia emissions from animal manure reduce air quality. In particular, urinary N is rapidly converted to NH_3 during manure collection or storage and then to volatile nitrous oxide. However, faecal N is thought to be environmentally less harmful form of N, as it is more likely to contribute to the accumulation of soil OM than urinary N. As a consequence, the rate of NH_3 volatilisation from urine-rich manure is greater than the rate of NH_3 volatilisation from faeces-rich manure. Second, the N from urine leaching to the soil creates rapid N contamination in ground water (Varel *et al*., 1999).

Our results show that the CT in sainfoin, when fed as fresh forage or as WS can generate beneficial environmental effects in terms of N excretion without negative effects on N retention by ruminants.

Gas production

It is possible to measure *in vivo* CH_4 production using the dilution of an inert tracer gas, fluorine hexafluoride, introduced into the rumen (Johnson and Huyler, 1994).
We measured CH_4 production only *in vitro* (Experiment 2) using the gas production method of Theodorou *et al*., 1994.

A close correlation was observed between an increase in gas production due to PEG-inactivated CT and biological activity of CT content (HCL-butanol method) (**Figure 23**). The correlations were R^2=0.972 and 0.913 for the early (3.5 h) and the late fermentation period (24 h), and R^2=0.925 and 0.976 for the biological activity of CT or HCl- butanol

results. The decrease in gas due to active CT depends not only on the biological activity of CT but also on CT content as illustrated in **Figure 23** below.

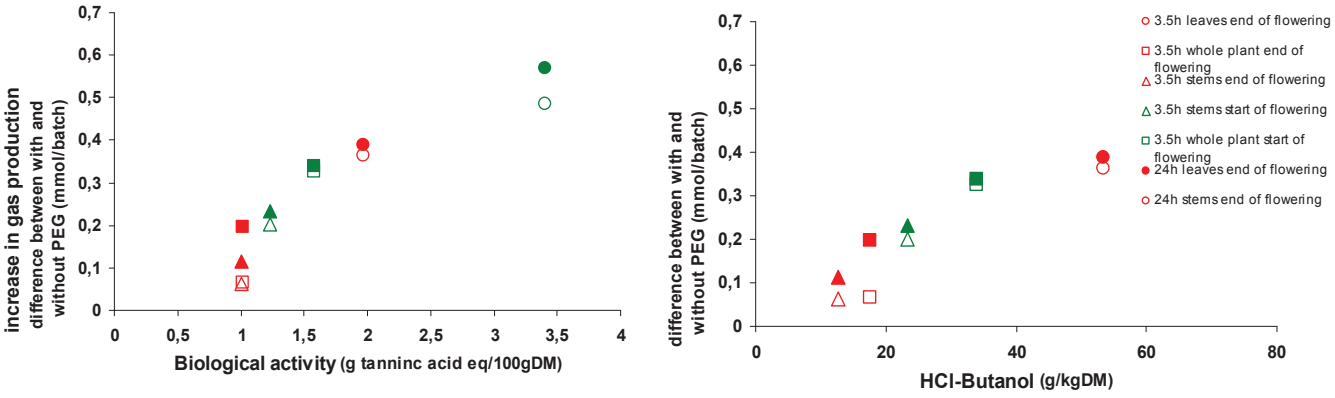


Figure 23: Relationship between the difference in *in vitro* gas production in the presence or absence of PEG and biological activity or CT content (HCl-butanol) of sainfoin CT.

CT in the leaves and stems decreased CH_4 production in the *in vitro* study after 3.5 and 24 h. The lower CH_4 production caused by sainfoin CT could be due to their anti-methanogenic activity and their interaction with rumen bacteria (McAllister *et al*., 1994, Puchala *et al*., 2005, Martin *et al*., 2010). The higher gas and CH_4 production observed for stems than leaves consistent and could be explained by their different CT composition (PD and *cis* value) and biological activity (**Figure 24**).

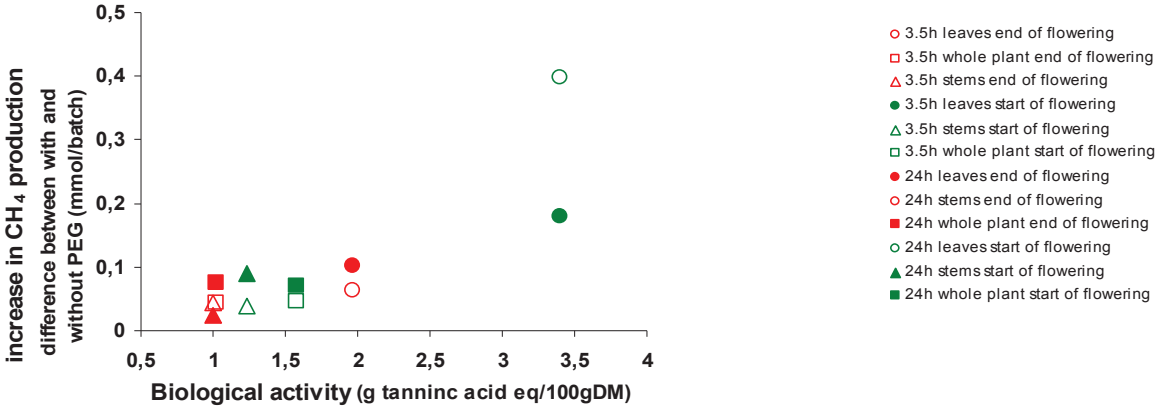


Figure 24: Relationship between *in vitro* CH_4 production and biological activity of sainfoin CT.

Conclusion

Although CTs in sainfoin seem to reduce rumen degradability of dry matter, the total tract digestibility is not reduced. This indicates that CT in sainfoin do not affect the energy value sainfoin when fed either fresh or as conserved, wrapped silage bales.

The CTs decrease the total N tract digestibility in relation with CT content, biological activity and structure. Our study confirms results of the literature which indicate that CT decrease ruminal N degradability. The difference in N degradability between stems and leaves in the rumen and in the intestine can also be explained by differences in biological activity and structure (mDP, PD and *cis* value). Despite decreased N digestibility, CT did not lower N retention as there was more N digested in the intestine and less N loss in the urine in the presence of active CT.

Sainfoin can be used to reduce the environmental impact of ruminants by reducing urinary excretion and methane emission. Decreasing the production of methane emissions from ruminants, by using tanniniferous plants without altering their nitrogen and energy value, is desirable as a strategy for reducing global greenhouse gas emissions and as a means for improving feed conversion efficiency.

III. THE NUTRITIVE VALUE OF SAINFOIN

In this part we bring together the different parameters of nutritional value, which were measured *in vivo* and enzymatic digestibility measurements, which enable predictiosn of *in vivo* digestibility. Our data are compared with those of the literature, making a distinction between data available for sainfoin, other legumes and lucerne which is a tannin-free legume.

1. Energy value

The main factor of variation in the net energy content of feeds is the digestibility of their crude energy, which is closely linked to the organic matter digestibility (OMD). The OMD can be measured *in vivo* or estimated from simple criteria of chemical composition or by an enzymatic method (pepsin-cellulase digestibility).
The detergent method for determination of cell-wall constituents is not suitable for tannin-rich forages because artefacts arise due to tannins, proteins and their complexes in the fibre fractions (Makkar *et al*., 1995; Pagan *et al*., 2009). A treatment with sodium sulphite was therefore used on our samples and a model was established between untreated and treated samples. This model was applied to the results of NDF and ADF values of the literature to compare the results.

Comparison of OMD with other studied conducted on sainfoin

The OMD measured *in vivo* on fresh forage of Perly sainfoin at the first cycle (end of flowering stage) was comparable to the values listed in the Tables INRA (Baumont *et al*., 2007) for the same vegetation stage (607 *vs* 616). **Table 8** brings together the *in vivo* studies in sheep for sainfoin. The OMD measurements are often made on younger plants, and are difficult to compare with the values we obtained in the first or second cycles.

There is a lack of information in the literature on vegetation stage, variety and CT content and the method of CT assay and this does not allow the importance of each of these parameters on the OMD value to be accurately quantified. Taken together, the CT data obtained by the method of Terrill *et al*., 1992b, yield higher contents than measured with the HCl-butanol method modified for lower OMD values.

Prediction of OMD

As expected, for the three sainfoin varieties (n=39), pepsin-cellulase digestibility was closely correlated with NDF content (**Figure 25**). However, we note that the data for the Perly variety (n=3) diverge from the general relation, with a lower digestibility for the same NDF content. We cannot tell whether this is a specific feature of this variety, or an effect of growth conditions, which were different from those of the other varieties.

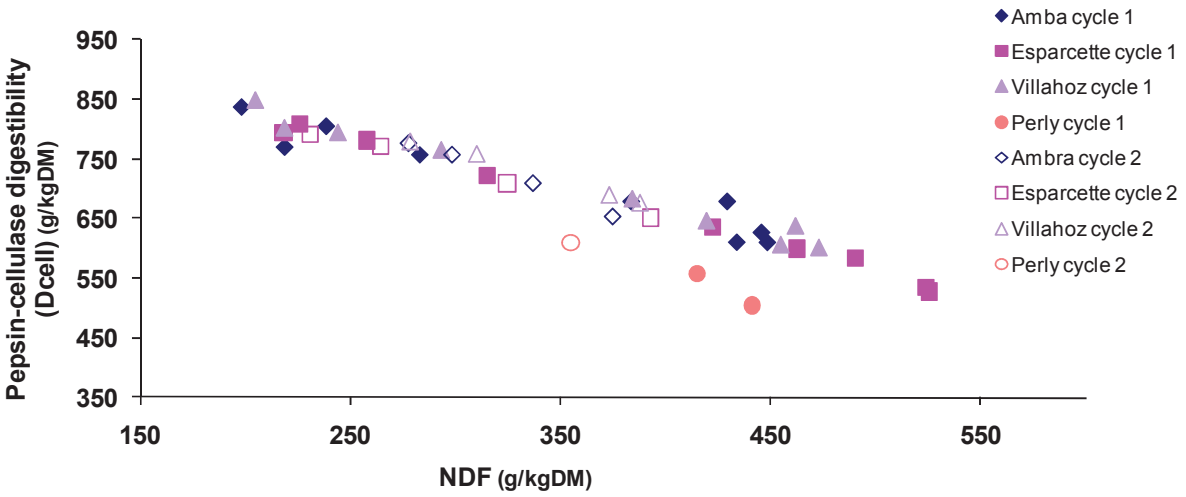


Figure 25: Relationship between pepsin-cellulase digestibility (Dcell) and NDF content for fresh forage of Perly and three other sainfoin varieties.

The OMD of legumes can be predicted from the pepsin-cellulase digestibility as previously stated. Ramirez-Restrepo *et al.*, 2006 have also shown that cellulolytic and hemi-cellulolytic enzymes can be used to predict the OMD of tannin-containing forage plants such as *Lotus corniculatus.*

The equation proposed in 2007 for predicting the OMD of fresh legume forage was based on 32 samples of lucerne and red clover (Aufrère *et al.*, 2007). We first tested this equation on our sainfoin samples, which were measured *in vivo,* and then constructed an equation with the eight sainfoin samples measured *in vivo* in the laboratory between 2004 and 2010 (**Figure 26**). The accuracy of the prediction was very high with this equation (R^2=0.936, RSD=1.41). The use of a specific equation for sainfoin appears justified insofar as the equation constructed in 2007 systematically underestimates the digestibility of sainfoin (**Figure 26**). This for the same enzymatic digestibility, the OMD of sainfoin was greater than that of lucerne and red clover. This difference can be explained by the lower NDF content of sainfoin, as we observed in Experiment 4. In

addition, according to the literature, the content of water-soluble lipids is higher for the sainfoin than for lucerne which can explain its higher digestibility.

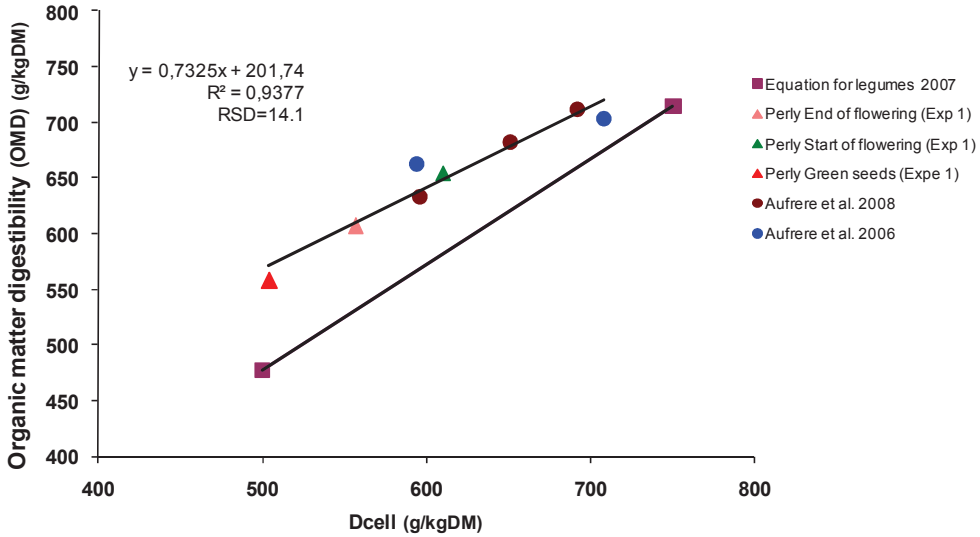


Figure 26: Relationship between OMD and Dcell for sainfoin and legumes.

In the text below, we use the OMD predicted for the three varieties of Experiment 4 using this equation constructed for sainfoin. Among these three sainfoin varieties which were grown under the same conditions, Esparcette had a lower OMD than Ambra and Villahoz. The OMD of fresh lucerne forage, which was predicted by fresh legume forage equations (Tables INRA, 2007) was lower than that of the three varieties of sainfoin.
Data from our experiments and the literature can be plotted on the same graph that illustrates the relationship between OMD and NDF (total n=57).

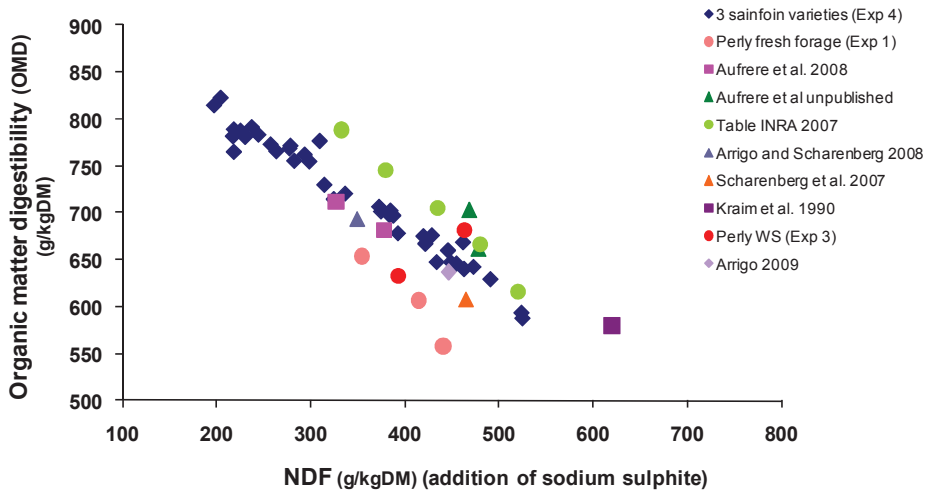


Figure 27: Relation between OMD calculated from pepsin-cellulase digestibility for the three varieties, or measured for Perly variety and literature values, and NDF content.

There is an overall correlation between OMD and NDF content, but the graph also reveals differences between varieties and between studies (**Figure 27**). Thus for the same NDF content Perly variety has a lower OMD. In contrast, data from studies listed in the Tables INRA 1989 show a higher OMD. The data from the study of Aufrère *et al*. (2008) are in line with the relation obtained for the three varieties also studied at Theix. Clearly, we lack explanatory information needed to interpret these differences, and the literature data are too scant to be of any assistance.

The system of forage units (UFL and UFV) expresses the energy values of forages in terms of their net energy content. For the fresh Perly sainfoin forage, which was harvested at the end-of-flowering stage (**Table 9**), the UFL value of 0.69 UF/kg DM was comparable to that in the Tables INRA, 2007 (0.70) and was lower than that obtained by Chassagne and Chambon (1993) in sainfoin harvested at the start-of-flowering stage (0.80).

Comparison with other tanniniferous plants

The most studied, the tannin-containing plants that are *Lotus corniculatus*, *Lotus pedunculatus*, sulla (*Hedysarum coronarium*) and several tropical plants (A*cacia, Desmodium, etc*.) as shown in **Table 10**. Some comparisons could not be made, in particular with study on acacia (Ben Salem *et al*., 1997, 2005) and some on sulla (*Hedysarum coronarium*) (Terrill *et al*., 1992), because these plants were studied in mixtures with other forages or with feed supplements.

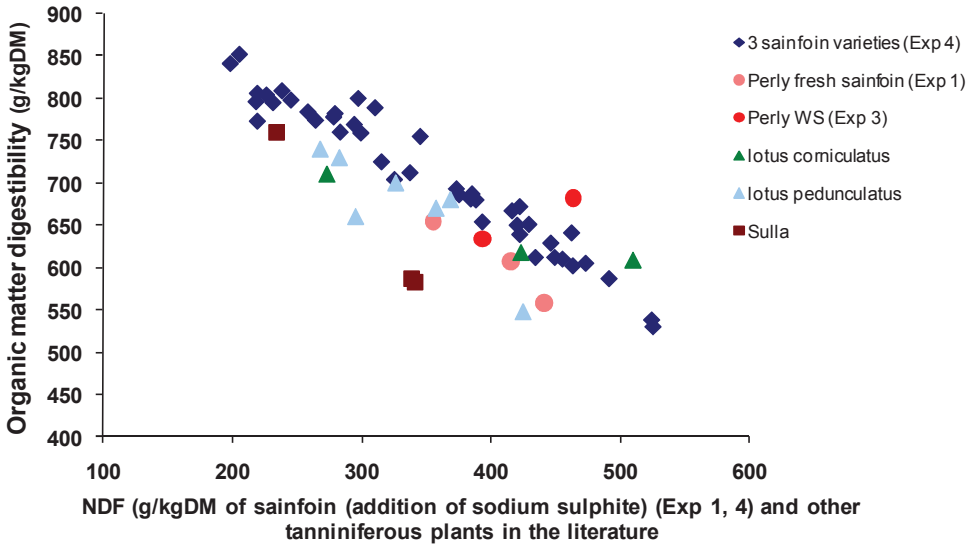


Figure 28: Relationship between OMD values and NDF content for our sainfoin results and those for other tanniniferous plants.

Despite the variability of CT content among these tanniniferous plants, the correlation between OMD and NDF content was surprisingly close (**Figure 28**). The fact that the OMD of sulla (600) was lower than the other tanniniferous plants may be related to the n-alkane method used for OMD measurement in this study (Mole *et al*., 2009). In the Tables Options Mediterranean (1990) we retained only the end-of-flowering sulla hay whose NDF content was given in the tables. Two fresh forages of sulla at the early and end-of-flowering stages had higher digestibilities, of 746 and 643, respectively, which could not be compared with other tanniniferous plants for lack of NDF values. In addition, the OMD of one sample of *Lotus pedunculatus (*Barry and Duncan, 1984) was low (547) as it contained a high concentration of CT (106 g/kg DM), which depressed the OMD (Waghorn *et al*., 1999). The equation linking OMD to NDF content in *Lotus* seemed to have the same slope as sainfoin.

In this comparison the CT contents of sainfoin measured by the Terrill method (whether modified or not) were lower (**Table 10**) than those of *Lotus* measured by vanillin assay. The degree to which these modifications were due to the environment cannot be quantified in this comparison.

Comparison with CT-free plants, e.g. lucerne

Lucerne, which makes up 80% of legumes in France, was chosen as a 'control' plant containing no tannins. OMD did not differ between sainfoin and lucerne (Kraiem *et al*., 1990, Aufrère *et al*., 2008) or among lucerne, birdsfoot trefoil and sainfoin when compared as hays or silage harvested at the early flowering stage (Kraiem *et al*., 1990) (**Table 11**).

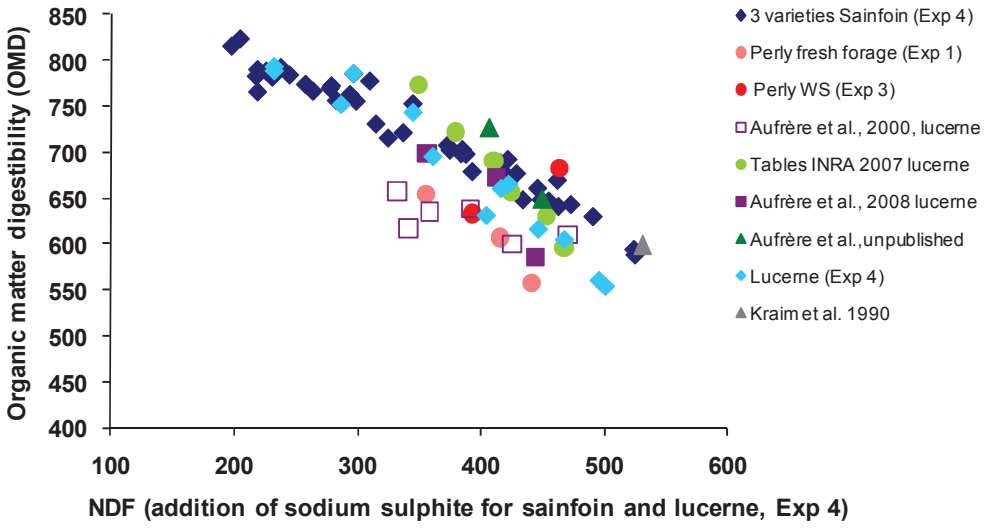


Figure 29: Relationship between OMD and NDF for sainfoin (Experiment 1, 3, 4), lucerne (Experiment 4) and results for lucerne from experiments described in the literature.

There was a close correlation between OMD and NDF for sainfoin and lucerne, for our results (n=57) (Experiment 4). Our samples of sainfoin and lucerne were treated with sodium sulphite, whereas a model was applied to the results for NDF values in the literature (n=18) in order to compare all the results. As sainfoin CT had no effect on OMD, the relation between OMD and NDF was the same for sainfoin and lucerne (**Figure 29**).

2. Nitrogen value

In the French PDI system (true Protein Truly Digestible in the small Intestine), the prediction of the PDI content of feed needs allowance for:

- Total nitrogen content (Nt)
- Theoretical degradability of nitrogen (DisN)
- True digestibility of undegraded dietary protein in the small intestine (dr)
- Fermentable organic matter (FOM) content

This last parameter, FOM, is calculated from the OMD retained to predict energy content.

Comparison of total tract nitrogen digestibility and ruminal nitrogen degradation with literature values for sainfoin

In the literature the few published values of nitrogen content for sainfoin were obtained using radio-labelled or non-radioactive tracers, and nylon bag degradability was measured in only two or three experiments.

The total tract N digestibility of the Perly variety, in the form of fresh forage, at the end of flowering was measured *in vivo* and was similar to the values listed in Tables INRA, 2007 for the same phenological stage at the first vegetation cycle (601 *vs.* 622). Also, in the second vegetation cycle, the values for total tract N digestibility were close to the same values given in the Tables INRA, 2007 (665 *vs.* 680). For all the literature results for sainfoin (**Table 9**) (n=19), DisN was weakly correlated with CP content (R^2=0.474).

According to Tables INRA (1989), the ratios between rumen degradable N and digestible organic matter (DOM) estimated (using DisN values) in Experiment 1 were 23.0, 23.2 and 24.4 for the regrowth, end of flowering and green seeds stages, respectively. In the PDI system, the optimal ratio is close to 20.2 g N/kg DOM to ensure microbial synthesis. Thus the ratio found with sainfoin allows microbes to use feed degradable N efficiently to produce their own proteins per unit of available energy.

Using our and literature data, we evaluated whether the ruminal nitrogen degradability (DisN) could be linked to forage constituents (**Figure 30**). In our study DisN was not

correlated with the CP content. For non-tannin-containing forages, including legumes like lucerne, Nozières *et al*. (2007) analysed a database of 375 measurements and obtained a good prediction of DisN by CP (R^2=0.87, RSD=3.5).

Neither was the DisN of sainfoin in the rumen correlated with total tract N digestibility or CT content. On the other hand, as we indicated above, the DisN of Perly sainfoin Perly was correlated with the biological activity of CT and some of CT structural parameters, which have been shown to play an important role in nutritional value for *Lotus corniculatus* and *Lotus pedunculatus* (Waghorn and Shelton, 1997).

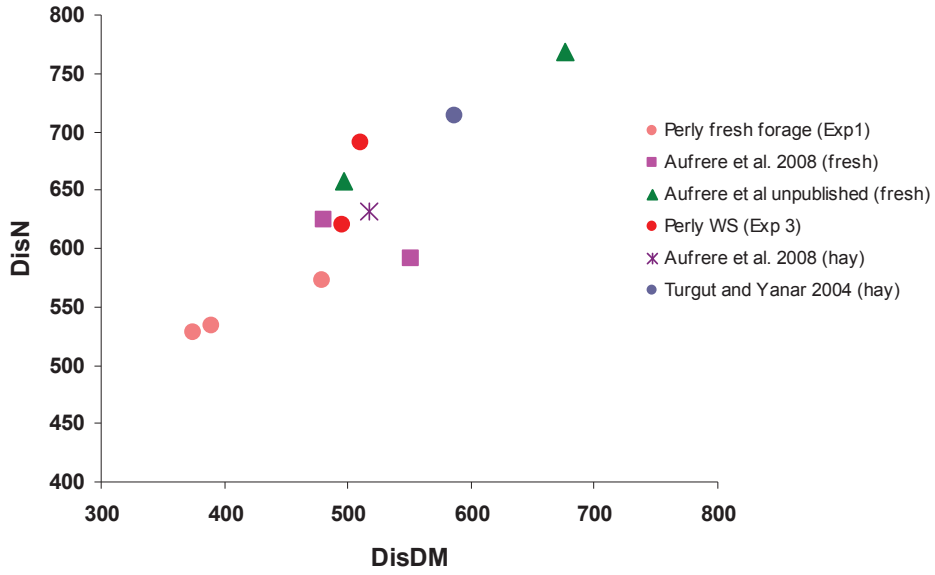


Figure 30: Relationship between DisN and DisDM for our results and those exist in the literature on sainfoin (For the Perly variety, DisDM and DisN for the whole plants were calculated from measurements made on leaves and stems).

However, a positive correlation was observed between DisN and DisDM (R^2=0.779, n=11) as observed on birdsfoil trefoil (Julier *et al*., 2003), and for most types of forages. Thus for tanniniferous forages, the DisN should mostly be determined by the effect of CT by the cell-wall or total dry matter degradation in the rumen.

DisN of CT-containing plants

It is well known that ruminal N degradation is lower for tanniniferous plants (Jones and Mangan, 1977). Nitrogen digestibility is also always lower than that for similar, but tannin-free forages. CT had an effect on CP and dry matter degradation even when the tannin content was low, as observed by Hedquist et al. (2000). The effect of CT on protection from degradation in the rumen as assessed by DisN, agreed with the findings of Arrigo and Scharrenberg (2008) who compare chicory, birdsfoot trefoil, and a mixture of gramineous legume with sainfoin. The same observations were also made by Aufrère *et al*., 2008 and Aufrère *et al*., (unpublished) on the comparison of DisN of sainfoin with lucerne. By contrast, Turgut and Yanar (2004) found the same DisN for sainfoin and lucerne. In the results of Julier *et al*., 2003, the CP degradation after 2 h and 8 h was greater in lucerne than in clover, birdsfoot trefoil and crownvetch. A comparison between three legume species native to Texas (*Acacia angustissima, Desmodium paniculatum* and *Lespedeza procumbens)* indicated that there were no differences in the N proportion remaining at 24 h and 48 h in the native legumes despite differences in protein-bound CT proportions remaining at those same times (Pawelek *et al*., 2008).
Studies by Reed (1995) indicated that the increased levels of CT in the forage can be nutritionally non-uniform and that the true digestibility of N cannot be predicted by regression analysis.

Degradation of N in the intestine

We know of only a few studies that estimated nitrogen digestion for tannin-containing forages in the intestine by the mobile bag method. In our study (Experiment 1) (mobile bag), in the presence of active CT, the N from leaf residues, which disappeared in the small intestine (SI) was enhanced due to increased amounts of N entering the SI. Aufrère *et al*., 2008 observed that the digestibility of rumen undegraded forage N in the SI was low with sainfoin and lucerne and much lower for sainfoin than for lucerne, while Riestra *et al*., 2010 observed similar coefficients for ruminal and intestinal disappearance of CP for *Desmodium paniculatum* and *Acacia angustissima*.
Comparisons of sainfoin, lucerne (Kraim *et al*., 1990) or white clover (Egan and Ulyatt, 1980) showed a similar N disappearance in SI among these species. Kraiem et al., 1990 also found no difference in N digestion between iso-nitrogenous *Lotus corniculatus* and lucerne, showing that CT in lotus had no beneficial effect. Conversely, Bermingham *et al*., 2001 reported that N disappearance in SI was lower for sainfoin than for sulla (*Hedysarum coronarium*). Other studies comparing sainfoin with lucerne or white clover

showed an increase in the digestion of nitrogen in the intestine or retained nitrogen (Waghorn *et al*., 1990).

In the French PDI system, the estimation of the true digestibility in the small intestine requires not only information on DisN but also on undigested dietary protein in the SI (PANDI). These values can be estimated using the the mobile bag method in the intestine. The quantity of residual proteins contained in the bags was analysed (Vérité *et al*., 1987) and can be considered as the PANDI. To compare our results with the values of the Tables INRA 2007, we calculated *in situ* N digestion in the small intestine (dr) from DisN and residual proteins measured in the mobile bags (PANDI). There was little difference between this dr value and the dr value calculated from the model of Nozières *et al*., 2007. DisN values were retained from our nylon bag measurements in the rumen. The revealed that the DisN values obtained from Table INRA 2007 which was calculated from CP, were not valid for tannin-containing forages.

Compared to the INRA Tables 2007 (**Table 9**), the PDIA (Dietary Digestible Protein in the Intestine) values for Perly sainfoin was 35 g/kg DM *vs* 30 for the end of flowering and 44 g/kg DM *vs* 33 for regrowth. The PDIN value (PDIA + PDI supplied by microbial protein from rumen degraded dietary protein) was 74 *vs*. 84 and 101 *vs* 103, and the PDIE value (PDIA+PDI supplied by microbial protein from rumen fermented organic matter) was 77 *vs* 76 and 88 *vs* 83, respectively. In our results, the DisN was lower than that in the Tables INRA 2007. Although there was more non-degradable CP arriving in the intestine, the coefficient of the dr was lower, the values of PDI were similar. On the other hand, because of the low value of DisN, the values of PDI and so therefore of PDIN were lower. Only the fresh Perly forage in the second cycle at start of flowering and WS had excess PDIN relative to their PDIE value. The nitrogen value increased with the energy value, as could be expected. Compared to lucerne at the same phenological stage, OMD of sainfoin was similar and the balance between PDIN and PDIE was better.

Conclusion

The OMD and therefore the energy value was closely linked to the NDF content, and so to the phenological stage for sainfoin as with other plants, whether or not they contain CT. The presence of CT in moderate quantity caused few changes in OMD, as previously observed. In contrast, as far as the nitrogen value is concerned, CT reduced

ruminal protein degradation and increased or decreased the intestinal digestibility. However, in general there was no direct link between CT content and protein degradation. The structure and biological activity of CT, and the chemical composition of sainfoin were important factors that influenced protein degradation. From DisN and non-degraded CP in the intestine, new PDI values were calculated. The PDIE value obtained for the end of flowering stage was similar to that in the Tables INRA, 2007 for the same vegetation stage, whereas the PDIN value was slightly lower.

Table 8: Chemical composition and parameters of digestion for sainfoin (*Onobrychis viciifolia*) from our study and from experiments described in the literature.

References	Specie	Preservation	Variety	Stade	Cycle	Method CT	CT	CP	OMD	Total tract	DisN	DisDM	NDFc	ADFc
							(g/kgDM)	(g/kgDM)	mesured	N digestibility				
Theodoridou et al. 2010	Sainfoin	fresh	Perly	end of flowering	1	Hcl butanol modified	17,6	143	607	601	534	390	415	314
Theodoridou et al. 2010	Sainfoin	fresh	Perly	green seeds	1	Hcl butanol modified	25,9	145	558	590	528	375	441	338
Theodoridou et al. 2010	Sainfoin	fresh	Perly	start of flowering	2	Hcl butanol modified	33,9	187	654	665	573	480	355	279
Theodoridou et al. 2010	Sainfoin	WS	Perly	end of flowering	1	Hcl butanol modified	25,6	140	682	548	691	510	463	350
Theodoridou et al. 2010	Sainfoin	WS	Perly	start of flowering	2	Hcl butanol modified	33,6	174	633	591	620	495	393	310
Aufrère et al. 2008	Sainfoin	fresh	Zeus	vegetative	1	Terrill et al. 1992	35,0	161	712	556	591	551	327	243
	Sainfoin	fresh	Zeus	start of flowering	1	Terrill et al. 1992	25,0	124	682	523	625	481	378	298
Aufrere et al unpublished	Sainfoin	fresh		vegetative	1	Terrill et al. 1992	64,0	189	703	599	768	677	468	314
	Sainfoin	fresh		start of flowering	1	Terrill et al. 1992	52,0	131	662	567	658	497	478	338
Tables INRA 2007	Sainfoin	fresh		early bud	1			184	788	703				195
	Sainfoin	fresh		bud	1			159	745	662				243
	Sainfoin	fresh		start of flowering	1			143	705	633				291
	Sainfoin	fresh		flowering	1			131	666	618				332
	Sainfoin	fresh		end of flowering	1			133	616	622				363
Tables INRA 1978	Sainfoin	fresh		regrowth 6 weeks	2			168	640	680				
Bermingham 2001	Sainfoin	fresh		vegetative	1	Terrill et al. 1992		243	680 (1)	650				
Arrigo and Scharenberg 2008	Sainfoin	fresh			1	Terrill et al. 1992	48,0	123	693	587	572		350	
Ulyatt and Egan 1979	Sainfoin	fresh	Fakir	vegetative	1			212	759					
Kraim et al. 1990	Sainfoin	silage+hay		start of flowering	1	Burns 1963	3,7	105	580	582			620	486
Scharenberget et al. 2007	Sainfoin	silage	Visnovsky		2	Terrill et al. 1992	71,0	191	608				465	387
Arrigo Y. 2009	Sainfoin	silage	Visnovsky	start of flowering		Terrill et al. 1992	44,2	144	637 (2)				447	412
Scharenberget et al. 2007	Sainfoin	dehydrated	Visnovsky	sown in May,harvested July	1	Terrill et al. 1992	77,0	195	675				575	520
Aufrère et al. 2008	Sainfoin	hay	Zeus	start of flowering	1	Terrill et al. 1992	6,0	90	633	464	631	517	444	352
Hanoglu H. 2004	Sainfoin	hay		mid flowering									474	
Turgut and Yanar 2004	Sainfoin	hay		early bloom								587		

(1): DMD

(2): *in vitro* estimation

Table 9: Nutritive value of sainfoin used in our study and from Tables INRA and of lucerne

	Sainfoin *(Onobrychis viciifolia)*							Lucerne	
	Tables INRA 2007	**Tables INRA 1989**	**Perly**	**Perly**	**Perly**	**Perly**	**Perly**		
	end of flowring	regrowth (a)	end of flowring	green seeds	regrowth (start of flowering)	WS end of flowering	WS regrowth (start of flowering)	Flowering	regrowth
CP(g/kg DM)	133	164	143	145	187	140	174	168	222
DisN	740	764	534	528	573	691	620	770	810
PIA	39	43	74	76	89	48	73	35	40
dr	770	760	467	524	496	178	391	810	840
PDIA	30	33	35	40	44	9	29	35	40
PDIMN	54	70	40	40	57	53	58	72	101
PDIME	47	50	42	36	44	35	41	45	50
PDIN	84	103	74	80	101	62	87	107	141
PDIE	76	83	77	76	88	44	69	80	90
OMD	620	680	610	560	650	540	620	600	680
UFL/kg DM	0,70	0,76	0,69	0,60	0,74	0.56	0,70	0.69	0.82
UFV/kgDM	0,61	0,68	0,60	0,50	0,66	0.46	0,61	0.59	0.75

(a) DisN and dr calculated with the model of 2007

Table 10: Chemical composition and parameters of digestion for tanniniferous plants from experiments described in the literature

References	Specie	Preservation	Variety	Stade	Cycle	Method CT	CT	CP	OMD	Total tract	NDFc	ADFc
								(g/kgDM)	measured	N digestibility		
Wang et al. 1994	Lotus corniculatus	fresh				Terrill et al. 1992	35	218	774			
Chiquette et al. 1989	Lotus corniculatus	fresh		vegetative	2	vanillin	32	151	689	699		332
Kraim et al. 1990	Lotus corniculatus	hay+silage				Burns 1963	13	159	608	693	509	411
Douglas et al.1999	Lotus corniculatus	fresh	Goldie	vegetative	1	Terrill et al. 1992	50	188	710		273	236
Luque et al. 2000	Lotus corniculatus	fresh	Goldie			Terrill et al. 1992	23,7	148	714			
Tables mediterranéennes 1990	Lotus corniculatus	hay						167	617	628	423	376
Barry et Manley 1984	Lotus pedunculatus	fresh	Maku	vegetative		vanillin	46	258	730	710	282	246
	Lotus pedunculatus	fresh	Maku			vanillin	106	198	660	560	295	254
Barry et al. 1986	Lotus pedunculatus	fresh	Maku		2	vanillin	95	193	670	580	357	303
Waghorn et al. 1994	Lotus pedunculatus	fresh	Maku	vegetative+flowers		vanillin	55	238	DM 680	670	326	272
Waghorn et Shelton 1995	Lotus pedunculatus	fresh		vegetative		vanillin	52	179	720(DM)	653	267	238
	Lotus pedunculatus			reproductive phase		vanillin	53	152	658(DM)	488	369	291
Barry et Duncan 1984	Lotus pedunculatus	fresh	Maku	vegetative	1	vanillin	63	158	547	479	425	274
Douglas et al. 1999	Sulla(Hedysarum coronarium)	fresh	Aokau	vegetative		Terrill et al. 1992	72	163	760		234	233
Bermingham et al. 2001	Sulla(Hedysarum coronarium)					Terrill et al. 1992	64	214	580	700		
Stienezen et al. 1996	Sulla(Hedysarum coronarium)	fresh		mid flowering	2	Terrill et al. 1992	72	188	705	631		
Molle et al. 2009	Sulla(Hedysarum coronarium)	fresh		flowering				194	583 (DM)		341	288
Tables mediterranéennes 1990	Sulla(Hedysarum coronarium)	hay		full bloom				143	587	557	339	276
Tables mediterranéennes 1990	Acacia cyanophylla (leaves)	fresh						186	433	605	421	210
Tables mediterranéennes 1990	Acacia cyanophylla (twigs)	fresh						148	510	330	265	288
Barahona et al. 1997	Desmodium ovalifolium	sun dried				Terrill et al. 1992	194	169	513	549	598	468
Carulla et al. 2001	Desmodium ovalifolium	fresh				Terrill et al. 1992						
Carulla et al. 2001	C macrocarpum	fresh				Terrill et al. 1992						
Barahona et al. 1997	Flemingia macrophylla	sun dried				Terrill et al. 1992	349	231	453	486	547	435
Vitti et al. 2005	cajanus cajan					Biological activity	0,5	170	420(DM)	635		
	Leucaena leucocephala					equivalent catechins	12,7	153	364	497		
	sesbania sesban						23,5	153	318	670		
Turgut and Yanar 2004	common vetch	hay		beginning lower pods				125				
Hanoglu H. 2004	common vetch	hay						206			359	282

Table 11: Chemical composition and parameters of digestion for lucerne from experiments described in the literature

References	Specie	Preservation	Variety	Stade	Cycle	CP	OMD	dMAT	DisN	DisDM	NDFc	ADFc
						(g/kgDM)						
Kraim et al. 1990	lucerne	hay+silage		start of flowering	1	145	599	732			531	430
Aufrère et al. 2000	lucerne	fresh		vegetative	1	201	657		800	645	332	200
		fresh		bud	1	173	600		761	568	425	279
		fresh		stemmy regrowth	2	184	638		810	588	391	281
		silage		bud	1	180	635		838	578	358	280
		silage +Ac Form		bud	1	180	617		804	568	341	261
		hay		bud	1	175	610		664	453	470	326
Turgut and Yanar 2004	lucerne	hay		early boom	1	108			699	506		
Tables INRA 2007	lucerne	fresh		vegetative (30 cm)	1	246	774	810			349	215
	lucerne	fresh		vegetative (60 cm)	1	225	722	784			379	241
	lucerne	fresh		early bud	1	206	690	774			410	270
	lucerne	fresh		bud	1	193	656	761			424	282
	lucerne	fresh		start of flowering	1	178	630	742			453	311
	lucerne	fresh		flowering	1	168	596	729			467	312
Aufrère et al. 2008	lucerne	fresh	Aubigny	vegetative stage	1	196	698	714	832	658	356	223
		fresh	Aubigny	bud stage	1	175	673	728	804	553	412	287
		Hay	Aubigny	vegetative stage	1	128	585	683	767	454	444	331
Aufrère et al unpublished	lucerne	fresh	Aubigny	vegetative stage	1	227	726	784	857	667	407	244
		fresh	Aubigny	bud stage	1	197	649	737	819	616	449	298

Conclusions and Perspectives

Conclusions and Perspectives

The aim of this research and PhD thesis were to add to our knowledge of i) the nutritional value of the sainfoin fodder legume and ii) the environmental benefits that can be derived from this ruminant feed (in terms of N excretion and gas emissions). These aims were studied by developing a fuller understanding of the effects of the condensed tannins (CT) in sainfoin.

This study confirmed that the impact of CT on digestion and the nutritive value of forages depends on CT content, structure and biological activity. Our results demonstrate that there is a close relationship between the CT content and biological activity, and that the main parameters of CT structure are correlated. The CT characteristics vary according to the phenological stage and the variety. For the whole sainfoin plants, CT content tended to increase with the phenological stage. For the three sainfoin varieties (Ambra, Esparcette and Villahoz), prodelphinidin and mean degree of polymerisation values increased with the phenological stage, and this was related to the decrease in biological activity. The CT characteristics of the Perly variety appeared to differ from the three other varieties. However it is not clear whether these differences were due to a variety effect or an environmental effect (soil, temperature, altitude) as the Perly variety grown under different environmental conditions.

In agreement with the literature, we could confirm that the structural complexity of CT and the methodological problems of their analysis make it difficult to compare between results. Therefore, the 'HealthyHay' project used the thiolytic tannin degradation method in order to overcome the limitations of colorimetric assays. This study found that the thiolysis results were lower but there was a close correlation between the thiolysis and the HCl-butanol.

In our study, the highest CT content measured by the thiolysis method was observed for Esparcette (6.05 gCT/kg DM), which may explain its lower digestibility compared with the other two sainfoin varieties during the first vegetation cycle. Several authors have suggested that dietary contents of <50 gCT/kg DM are beneficial. However, these recommendations varied according to the analytical method used and originated mainly from feeding trials with *Lotus* species and may not be applicable to other feeds.

Our results demonstrated that CT in fresh sainfoin or wrapped sainfoin silage bales decreased total-tract N digestibility; but CT did not affect organic matter digestibility. We confirm the general reduction of ruminal protein degradation by CT and we shed some more light on the protein degradation of leaves and stems in the rumen and intestine and related thse to differences in CT content, biological activity and structure (mDP, PD and *cis*). CT in leaves and stems greatly increased the *in situ* estimate of forage N escaping from the rumen. CT also caused a higher N disappearance from leaves in the intestine.

In addition, CT maintain the ability to affect the nutritional value of preserved sainfoin, although the biological activity in wrapped sainfoin silage bales was reduced. Unlike tannin-free legumes, sainfoin may be easier to ensile as the presence of CT can prevent protein proteolysis during the ensiling process.

The experimental studies revealed that sainfoin generated significantly lower urinary N losses without negatively impacting on N retention. This is important in terms of ruminant nutrition. Also, our *in vitro* results showed that there is potential for using sainfoin in order to reduce CH_4 emissions from ruminants. It is suggested that tanniniferous plants such as sainfoin can reduce greenhouse gas emissions and nitrogen losses from ruminants without altering their nitrogen and energy values. This is advantageous as a strategy for reducing the environmental impact of forages and as a means of improving feed conversion efficiency.

The nutritive value of sainfoin is high, despite its lower nitrogen content than lucerne. In comparison with lucerne, a 'tannin-free forage' widely used in France (Table INRA, 2007), the OMD values of sainfoin are similar at the same phenological stages. Moreover, the balance between PDIN and PDIE was better for sainfoin than for lucerne at the same phenological stage.

Perspectives

Future research on the nutritional impact of CT must not only be based on CT content in plant tissue, but must also include structure-activity considerations.

Our experiments reported in this thesis give evidence that the content, structure and biological activity of CT change through the plant's life under different environmental conditions. The mechanism and environmental factors bringing about these changes are still not well known and future research is needed to identify them in relation to their nutritional impact.

Conventional plant breeding of sainfoin could be used to optimise its tannin content and so increase its nutritional value. Also, given some agronomic difficulties with growing sainfoin, one promising option could be the introduction of tannins into major forages like lucerne, and white and red clover, through genetic engineering.

In the light of new information, there is scope to further simplify the analytical methods for tannins and make them more accurate. Biological activity appears to be a relatively simple assay suited to assessing the activity of tannins in a large number of samples in breeding programs, for example. The use of NIRS, as we have begun to develop calibration equations, should make this possible.

My thesis may have some practical applications in ruminant nutrition and the production of tannin-containing forages.

The results we obtained with sheep at maintenance fed a diet containing only sainfoin need to be tested with producing animals, for example growing sheep and cattle, lactating ewes, goats or cows. In producing animals the diet will comprise different forage sources and supplement feeds.

Sainfoin is seldom used as a pure crop. Generally, it is introduced in pasture in a grass-legume mix with cocksfoot (*Dactyle*), ryegrass (*Lolium*) or with other legumes such as red clover, white clover or lucerne. Previously it has been found that 50-75% of sainfoin is the minimum amount in a mixture with lucerne to get significant effects of CT on the whole diet

(Aufrere *et al*., unpublished data). Our results show the choice of the variety is important. A variety like Esparcette, characterised by the highest CT content and biological activity, may provide beneficial effects with a lower proportion of sainfoin in a mixture with other legumes.

Also, preserving legumes as silage is a good way to provide an on-farm source of home-grown energy and protein, offers advantages over traditional haymaking, being less weather-dependent, and allows a high quality of forage during the harvesting period. In particular, wrapped silage bales of sainfoin have great potential in animal nutrition and can be used by farmers, as we found the CT effects were not reduced by this mode of conservation.

Publications

Publications
The following papers were produced from this work and are incorporated in this Thesis.

Scientific Publications

* **Theodoridou K**., Aufrère J., Andueza D., Pourrat J., Lemorvan A., Stringano E., Mueller- Harvey I., Baumont R. The effect of condensed tannins in fresh sainfoin (*Onobrychis viciifolia*) on *in vivo* and *in situ* digestion in sheep. **Animal Feed Science and Technology, 160, 23-38**.

* **Theodoridou K**., Aufrère J., Andueza D., Pourrat J., Picard F., Lemorvan A, Baumont R. The effect of condensed tannins in wrapped silage bales of sainfoin (*Onobrychis viciifolia)* on *in vivo* and *in situ* digestion in sheep. **Submitted in Animal.**

* **Theodoridou K**., Aufrère J., Andueza D., Lemorvan A., Picard F., Stringano E., Pourrat J., Mueller-Harvey I., Baumont R. Effect of plant development during first and second vegetation cycle on chemical composition, condensed tannins and nutritive value of three sainfoin (*Onobrychis viciifolia*) varieties compared to lucerne. **Submitted in Grass and Forage Science.**

* **Theodoridou K**., Aufrère J., Niderkorn V., Andueza D., Lemorvan A., Picard F., Baumont R. *In vitro* study of the effects of condensed tannins in sainfoin on the digestive process in the rumen, at two vegetation cycles. **Submitted in Animal Feed Science and Technology.**

Communications

* Stringano, E., Hayot, C., Smith, L., **Theodoridou, K.,** Aufrere, J., Brown, R., Hayes, W., Cramer, R., Mueller-Harvey, I. 2010. Thiolytic screening method for exploring condensed tannin variation in a unique sainfoin germplasm bank. **Planta Medica 76 (12) : 1166-1166.**

* **Theodoridou K**., Aufrère J., Andueza D., Pourrat J., Baumont R. 2010. Development and nutritive value of three varieties of sainfoin compared to lucerne during primary growth. **Proceedings of European Grassland Federation (EGF), 452-454, Kiel, Germany**.

* **Theodoridou K**., Aufrère J., Andueza D., Pourrat J., Baumont R. 2010. Nutritive value of sainfoin harvested at two phenological stages as fresh and preserved as wrapped silage bales. **Proceedings of European Association Animal Production (EAAP), 170 p, Crete, Greece.**

* Ojeda-Robertos N., Manolaraki F., **Theodoridou K**., Aufrère J., Halbwirth H., Stich K., Regos I., Treutter D. Mueller-Harvey, I.Hoste H. 2010. The anthelmintic effect of sainfoin (silage, hay, fresh) and the role of flavonoid glycosides. **Proceedings of European Association Animal Production (EAAP), 73 p, Crete, Greece.**

* Asma A. F., **Theodoridou K**., Halbwirth H., Aufrère J., Stich K. 2009. Effect of different conservation methods and maturity stages on peroxidase activity in sainfoin *(Onobrychis viciifolia).* **Proceedings of symposium 'Plant Phenolics for the Health of Plants, Humans and Livestock', 104 – 106 p, Freising, Germany.**

* **Theodoridou K**., Aufrère J., Andueza D., Baumont R. 2009. Use of polyethylene glycol (PEG) to assess the effect of condensed tannins on nitrogen balance and digestibility in sheep fed fresh sainfoin (*Onobrychis viciifolia*)**. Proceedings of XI International Symposium on Ruminant Physiology, 96 p, Clermont-Ferrand, France.**

* **Theodoridou K**., Aufrere J., Andueza D., Montet G., Baumont R. 2008. Comparaison *in situ* de la dégradabilité de la matière sèche et de l'azote de deux variétés de sainfoin, cultivées en deux lieux différents et conservées en fourrage vert et ensilage. **Proceedings of Rencontre Recherche Ruminants**, **15, p 287, Paris, France.**

* **Theodoridou K**., Aufrère J., Andueza D., Baumont R., 2011. Use of polyethylene glycol (PEG) to assess the effect of condensed tannins on nitrogen balance and digestibility in sheep fed fresh sainfoin (*Onobrychis viciifolia*), **3° Panhellenic Conference of Animal Production. Thessaloniki, Greece.**

* **Theodoridou K**. 2010. Effect of condensed tannins on the nutritive value of sainfoin (*Onobrychis viciifolia*). '**Journees de l'Ecole Doctorale'**, 6-7 March. Clermont-Ferrand, France.

* **Theodoridou K.** Effect of condensed tannins on the nutritive value of sainfoin (*Onobrychis viciifolia*). **Meetings in the 'Healthy Hay' network.**
Vienna: 4/11/2007, Vienna: 21/02/2008, Copengague: 6/11/2008
Munich: 15/03/2009, Reading: 18/11/2009.

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Abstract

The effects of condensed tannins in sainfoin (*Onobrychis viciifolia*) on its digestion and nutritive value

Proteins in forage legumes are often poorly utilized by ruminants because of their extensive, rapid degradation in the rumen, which leads to N losses via urinary urea excretion. One solution for improving protein utilisation is the use of forages that contain condensed tannins (CT) able to bind proteins and reduce protein degradation in the rumen. The aim of this thesis was to add to our knowledge of the nutritional value of the fodder legume sainfoin and on the environmental impact of its digestive utilisation by ruminants. The first objective was to investigate how the content, structure and biological activity of the CT in sainfoin varied with phenological stage, preservation method and variety. The second objective was to assess the effects of CTs on the digestive processes that determine nutritional value, nitrogen excretion and gas emissions by the animal.

Four experimental studies were conducted to address the questions raised. In the first *in vivo* experiment we studied the effect of CT in sainfoin at three phenological stages, used as fresh forage, on the sheep's digestive and nitrogen balance and on the ruminal and intestinal digestion measured *in situ* for leaves and stems. This *in vivo* study was extended by an *in vitro* one, designed to measure, on the same forages, the effect of CT on the production and composition of fermentation gases (CO_2 and CH_4). A second *in vivo* study was conducted on wrapped silage bales of sainfoin (WS), harvested at the same phenological stages as the fresh forage. To assess effects of sainfoin CT, measurements were made with and without polyethylene glycol (PEG), which inactivates CT. Lastly, we investigated differences among three sainfoin varieties cultivated in a common environment over the course of the first and second vegetation cycles and compared them with a lucerne variety also grown in the same location.

For the three sainfoin varieties (Ambra, Esparcette and Villahoz), CT content, prodelphinidins (PD) and mean degree of polymerisation (mDP) values tended to increase with the phenological stage in relation with a decrease in biological activity. By contrast, mDP and PD values of the Perly variety decreased with the phenological stage with no change in biological activity. In fresh or WS sainfoin CT decreased total-tract N digestibility but did not affect organic matter digestibility (OMD) and N retention. CT in leaves and stems greatly increased the *in situ* estimate of fresh forage. N escaping from the rumen and a higher N disappearance in the intestine was obtained for leaves. Sainfoin CT decreased urinary N losses and CH_4 production. Compared with lucerne at the same phenological stage, OMD of sainfoin was similar and the balance between PDIN and PDIE was better.

Future research on the nutritional impact of CT must not be based only on the CT content of plant tissue but must also include structure-activity considerations. Utilisation of tanniniferous plants such as sainfoin to reduce GHG emissions and N losses without altering their nitrogen and energy value is promising both as strategy to curb the environmental impact of forage utilisation and as a means of improving feed conversion efficiency.

Keywords: Sainfoin, condensed tannins (structure, biological activity), nutritional value, digestion, *in vivo* and *in situ* measurements (rumen, intestine), *in vitro* gas production, polyethylene glycol, phenological stage

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Résumé

Les effets des tannins condensés du sainfoin (*Onobrychis viciifolia*) sur sa digestion et sa valeur nutritive

Les protéines des légumineuses fourragères sont souvent mal utilisées par les ruminants en raison de leur importante solubilité et de leur dégradation rapide dans le rumen, qui conduit à des pertes importantes d'azote dans l'urine. Une solution pour améliorer l'utilisation des protéines est l'emploi de fourrages contenant des tannins condensés (TC) capables de se lier aux protéines et ainsi de réduire leur dégradation dans le rumen. Cette thèse avait pour but d'améliorer les connaissances sur la valeur nutritive du sainfoin, une légumineuse fourragère riche en TC, et sur les impacts environnementaux de son utilisation digestive par les ruminants. Le premier objectif était d'étudier l'évolution de la teneur, de la structure et de l'activité biologique des TC du sainfoin en fonction du stade phénologique, du mode de conservation et de la variété étudiée. Le deuxième objectif était d'évaluer les effets des TC sur les processus digestifs qui déterminent la valeur nutritive, l'excrétion d'azote et les émissions de gaz par le ruminant.

Quatre études expérimentales ont été menées pour répondre aux questions soulevées. Dans la première expérience *in vivo*, nous avons étudié chez le mouton l'effet des TC du sainfoin récolté en vert à trois stades phénologiques sur la digestion de la matière organique et de l'azote, et sur la digestion ruminale et intestinale mesurée *in situ,* séparément pour les feuilles et les tiges. Cette étude *in vivo* a été complétée par une deuxième expérience menée *in vitro* sur les mêmes fourrages afin de mesurer la production de gaz (CO_2 et CH_4). Une troisième expérience, réalisée *in vivo* chez le mouton, a permis étudier la digestion des fourrages de sainfoin de la première expérimentation conservés en ensilages mi-fanés. Les effets des TC du sainfoin ont été mis en évidence par l'addition de polyéthylène glycol (PEG), qui inactive les TC. Enfin dans une quatrième expérience, nous avons étudié, l'évolution au cours du premier et du deuxième cycle de végétation, des TC, de la composition chimique et de la digestibilité enzymatique pour trois variétés de sainfoin et une variété de luzerne, cultivées dans un même environnement.

Pour les trois variétés de sainfoin (Ambra, Esparcette, Villahoz), la teneur en TC, la proportion de prodelphinidines (PD) et le degré moyen de polymérisation (mDP) des TC augmentent avec le stade phénologique, alors que l'activité biologique des TC diminue. En revanche pour la variété Perly cultivée dans des conditions environnementales différentes, les valeurs de mDP et de PD diminuent avec le stade phénologique sans que l'activité biologique ne soit modifiée. Dans les études menées *in vivo* avec la variété Perly, les TC du sainfoin, utilisé en vert ou en ensilage mi-fané, diminuent la digestibilité de l'azote, sans affecter la digestibilité de la matière organique (DMO) et la rétention azotée. Les TC augmentent la quantité d'N des feuilles et des tiges, estimée *in situ*, qui échappe à la dégradation dans le rumen, ce qui conduit à une plus grande disparition de l'N dans l'intestin pour les feuilles. Les TC du sainfoin entraînent une diminution des pertes d'azote sous forme d'N urinaire mesurées *in vivo* et de la production de CH4 mesurée *in vitro*. A même stade phénologique la DMO du sainfoin est semblable à celle de la luzerne et l'équilibre entre les valeurs PDIN et PDIE est amélioré.

Les recherches futures sur l'impact nutritionnel des TC ne doivent pas seulement prendre en compte la teneur en TC, mais aussi les relations entre leur structure et l'activité biologique. Pour une même valeur énergétique et azotée que la luzerne, l'utilisation digestive du sainfoin entraîne moins de perte d'N dans l'urine, et pourrait entraîner une moindre émission de CH_4. Le sainfoin constitue donc une plante prometteuse qui, en améliorant l'efficacité de l'utilisation du fourrage permet de réduire son impact environnemental.

Mots-clés: Sainfoin, tanins condensés (structure, activité biologique), valeur nutritive, digestion, mesures *in vivo* et *in situ* (rumen, intestin), production de gaz *in vitro,* polyéthylène glycol, stade phénologique.