Effect of wilting, silage additive, PEG treatment and tannin content on the distribution of N between different fractions after ensiling of three different sainfoin (*Onobrychis viciifolia*) varieties

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Abstract

Sainfoin (*Onobrychis viciifolia)* is a tanniniferous, leguminous plant that has potentially beneficial effects on protein utilization in ruminants. Since ensiling causes protein breakdown and elevated levels of buffer soluble N (BSN), we studied the distribution of N before and after ensiling sainfoin. Three varieties of sainfoin were either direct-cut and frozen directly or wilted and frozen before later ensiling in mini-silos with and without acidification with Promyr (PM; an acidifying commercial mixture of propionic and formic acid) and with or without polyethylene glycol (PEG). Extractable tannins (ET) and protein bound tannins (PBT) were measured with an HCl/butanol method in an attempt to correlate tannin levels to N fractions. The sainfoin silages showed good ensiling characteristics and had relatively high concentrations of un-degraded protein. The effect of wilting on BSN levels (g/kg N) was dependent on sainfoin variety ($P<0.001$). PEG increased and PM decreased the level of BSN in the silages ($P<0.001$). PM treatment also produced less non-protein N and ammonia-N ($P<0.05$) as compared with no additive. Addition of PEG to the silage increased the BSN-proportion 1.8- and 2.6-fold for both DM stages. A strong tannin-protein binding effect is, therefore, confirmed in sainfoin. However, correlations between tannin levels (ET and PBT) and BSN were poor in the (non-PEG) silages, indicating either that the HCl/butanol method is unsuitable for measuring tannin in silages or that qualitative attributes of tannins are more relevant than quantitative. The HCl/butanol method seems therefore not to be useful to predict degradation of protein in sainfoin silages.

Keywords: Sainfoin, Legumes, Tannin, Silage, Protein, Nitrogen

1. Introduction

High producing dairy cows require high quality forages that can match their needs.. Forage quality is often associated with high protein content but protein quality is also of importance. Knowledge about factors influencing protein quality and methods to estimate it is of considerable importance for formulating cost effective and environmentally friendly rations to ruminants. One of the central issues in this area is the proportion of feed protein which breaks down in the rumen (Chalupa and Sniffen, 1996). However, breakdown of protein starts already during conservation of the forage as hay or silage. Ensiling forage is used as a means to conserve and maintain its nutritive value and has become increasingly important in the last decades. It is believed that the effect of tannins in sainfoin can reduce proteolysis which takes place in the silo (Albrecht and Muck, 1991; Salawu, *et al.*, 1999; Wilkins and Jones, 2000).

Wilting prior to ensiling reduces water content and therefore increases the concentration of sugars which is particularly important for legumes which are generally known to have low levels of sugars. Less storage requirements and a lower volume of silage effluents are further advantages of wilting before ensiling (McDonald, *et al.*, 1991). In some regions, where climate does not allow wilting, silage quality can be improved by acidifying the forage in order to decrease pH and thereby reduce proteolysis or clostridial growth.

The forage legume sainfoin (*Onobrychis viciifolia*) has specific benefits to ruminant protein nutrition (Karnezos, *et al.*, 1994; Majak, *et al.*, 1995; Caygill and Mueller-Harvey, 1999; Koivisto and Lane, 2001; Heckendorn, *et al.*, 2006). These benefits are believed to be the result of the presence of condensed tannins. Tannins can have a wide spectrum of beneficial - as well as detrimental - effects on the digestion of proteins and other feed components. Under certain conditions such as optimal pH or specific tannin:protein ratios, tannins have the ability to bind to proteins, making them unavailable to rumen microorganisms but without impairing their

digestion and absorption in the small intestine (McNabb, *et al.*, 1996; Wang, *et al.*, 2007). Also, a direct inhibition of microbial cell wall synthesis in the rumen, decreasing the microbial proteolytic potential, has been reported. (Jones and Mangan, 1977; Barry and Duncan, 1984; Jones, *et al.*, 1994). Lees (1992) points out that condensed tannin-containing legumes like sainfoin do not cause bloat from grazing in contrast to tannin-free protein-rich legumes such as alfalfa. For this reason, attempts to introduce genes into alfalfa that induce the synthesis of condensed tannins have been made (Tanner, *et al.*, 1997; Johnson, *et al.*, 2007 (U.S. Patent)). However, tannin containing plants could have detrimental effects in form of decreased voluntary feed intake or impaired carbohydrate digestion (Barry and Duncan, 1984).

Controversy exists on how to measure tannins in plants. Recent studies show that the protein-binding effects of tannins are influenced by many factors other than tannin concentration such as tannin molecular structure, their degree of polymerization, the ratio of proteins to tannins, protein structure and amino acid composition etc. (Spencer, *et al.*, 1988; Silber, *et al.*, 1998; Frazier, *et al.*, 2003; McAllister, *et al.*, 2005; Deaville, *et al.*, 2007). These questions have been reviewed by Aerts *et al.* (1999) and Mueller-Harvey (2006). Colorimetric methods like the HCl/butanol methods, originally from Porter (1992) and their modification, do by their nature not account for the qualitative characteristics listed above. They have therefore been questioned and also for the fact that colour yield is not always linear (Giner-Chavez, *et al.*, 1997a; Makkar, *et al.*, 1999; Schofield, *et al.*, 2001). However, the informative value of these methods will depend on plant species and maturity, choice of standard, sample extraction, preparation method, etc.. (Hagerman, 1988). Therefore, many different colorimetric methods and their modifications are still employed in attempts to predict ruminal protein metabolism (Jayanegara, *et al.*, 2009) and may, under the right conditions and within the same plant species, give reliable results and still be useful for ranking varieties within a certain species and may also allow comparison between

direct-cut, wilted and/or ensiled forage (Barry and Forss, 1983; Giner-Chavez, *et al.*, 1997b; Rubanza, *et al.*, 2005; MacKown, *et al.*, 2008; Rothman, *et al.*, 2009). Polyethylene glycol (PEG) has the ability to bind strongly to tannins and inhibits tannin-protein complex formation. This effect has been utilized to study if condensed tannins decrease protein degradation since PEG will limit the formation of protein-tannin complexes (Jones and Mangan, 1977; Makkar, *et al.*, 2007). The objectives of this paper were to study protein metabolism during ensiling of three different sainfoin varieties. The silage treatments tested were an acidifying additive and wilting. We also attempted to test for any effects of tannin level on protein breakdown and to compare the effects of tannin level and PEG on the distribution of N in consideration of the questioned applicability of the HCl/butanol method for sainfoin silage.

2. Material and Methods

2.1. Plant material

Plant materials of the varieties Cotswold Common, Reznos and Teruel were each collected randomly from the respective fields at CITA (Centro de Investigacion y Tecnologia Agroalimentaria) de Aragon, Spain, in late flowering stage, April/May 2007. The selected varieties were chosen because they were some of the few commercially grown varieties in Spain. Several samples of each variety were collected, pooled and split into two batches. One batch was wilted under natural field conditions to a dry matter (DM) content of approximately 500 g/kg and the other batch was directly frozen resulting in two DM stages. Samples were frozen and chopped into small pieces. A grass/clover (red clover) mixture (1:1) was harvested, wilted under natural field conditions and frozen at the Kungsängen Research Centre, Swedish University of

Agriculture in Uppsala, May 2008. The grass/clover sample, which was assumed to be free of tannins, was used as a "tannin blank". All sainfoin samples were frozen and transported to Sweden and either ensiled or freeze dried upon arrival.

2.2. Ensiling procedures

The following treatments were applied immediately before ensiling to each sample: a) PEG (100 g/kg dry matter), b) Promyr (2.5 g/kg fresh matter of Promyr® MT 570) and c) no additive. PEG (Merck, Darmstadt, Germany) had a MW of 3000 Da. Promyr consisted of a solution of >750 g/kg formic acid and sodium-formates in solution and <250 g/kg propionic acid (Perstorp Specialty Chemicals AB, Perstorp, Sweden). There were 12 silos of each of the 3 sainfoin cultivars and 12 silos containing grass/clover. From the 36 silos containing sainfoin, one third was treated with PEG, one third was treated with Promyr and one third was free of additives. The diluted additives were sprayed evenly over the plant material inside a plastic bag. The bag was closed and the content was thoroughly shaken in order to spread the additive evenly. Thereafter, the plant material was packed in glass silos (20 cm length and 3 cm inner diameter. After filling the silos with about 80 to 90 g, leaving approximately a 1-cm free headspace, the tubes were closed with a rubber stopper and a water lock. The silos were incubated in a dark 20°C room for 60 days. The ensiled material was frozen, freeze dried and ground on a "Brabender" cutter mill to pass a 1-mm screen prior to analysis.

2.3. Analytical methods

2.3.1. Dry matter, ash and N

All analyses were done on both ensiled and fresh material. Dry matter of fresh samples was determined by drying at 105°C to constant weight in a forced draught oven and for ensiled material by freeze drying and multiplication with a correction factor (0.94) for remaining water and volatile losses. All N analyses were done in duplicate on the freeze dried material by the Kjeldahl procedure using a Kjeltec Analyzer unit 2400 and a 2020 Digestor (Foss, Hillrød, Denmark) with Cu as a catalyst. Buffer soluble nitrogen (BSN) was determined by extracting freeze dried samples with a borate-phosphate buffer, pH 6.75, at 39ºC for 1 h according to a modified method by Licitra *et al.* (1996) as follows: 1.5 g of the freeze dried plant material was weighed into a 50-mL tube (Sarstedt, Nümbrecht, Germany) and mixed with 50 mL of the borate-phosphate buffer. The tubes were shaken and incubated for 1 h in a 39ºC water bath with an additional thorough shaking every 15 min. Thereafter, the tubes were centrifuged at 3000 *x g* on a swing-out rotor centrifuge (G4.11, Jouan, Saint Herbain, France). Twenty mL of the supernatant was transferred to a Kjeldahl tube and analyzed for BSN. To avoid floating particles, a polyester cloth (20-µm openings) was wrapped around the tip of the pipette and the liquid was pipetted slowly. A stepwise increase of the temperature of the Kjeltec digestion block was needed when analyzing the aqueous samples to prevent extensive foaming during digestion. Initially, temperature was slowly increased to evaporate excess water. In the last step, Cu-containing K_2SO_4 tablets were added and the standard procedure described above for solid samples was commenced. For analysis of non-protein N (NPN), another 15 mL of the BSN extract was transferred to a 30 mL polypropylene centrifuge tube and 1.5 mL of trichloroacetic acid (TCA; 200 g/L) was added and incubated for 1 h in ice water to precipitate polypeptides and proteins. After incubation, the sample was centrifuged at 27000 *x g* for 15 min in a spark proved Suprafuge 22 centrifuge with fixed-angle rotor (Heraeus Sepatech GmbH, Osterode, Germany) and the

168 aqueous supernatant was analyzed for the NPN according to the procedure for aqueous samples
169 mentioned above. The BSN fractions were expressed as proportions of total N.
170 Ammonia-N and α-amino acid N (AA-N) were analyzed using phenol-hypochlorite and
171 ninhydrin, respectively, on a Technicon Auto Analyser (Broderick and Kang, 1980). Leucine was
172 used as a standard for amino acids and ammonium sulphate (Merck, Darmstadt, Germany) as
173 standard for ammonia, respectively.
174
175 *2.3.2. Tannin analysis*
176 Tannin concentration was measured before and after ensiling according to a modification
177 of the method by Terrill *et al.* (1992). The original method was modified to cut down the use of
178 hazardous and/or expensive chemicals like mercaptoethanol and butanol.
179 A freeze dried sample (250 mg) was weighed into a 50-mL centrifuge tube (Sarstedt, Nümbrecht,
180 Germany) and extracted twice with 10 mL acetone:H_2O in ratio of 7:3 (v/v) with 1 g ascorbic
181 acid/L plus 10 mL of diethyl ether for 20 min in an ultrasonic ice water bath. The tubes were
182 centrifuged at 26000 *x g* for 15 min, the supernatants, which contained the extractable tannin
183 (ET) fraction, carefully decanted and combined in 50 mL tubes. The bright green, upper organic
184 phase was removed by suction and water with 1 g ascorbic acid/L was added to make up a
185 volume of 50 mL. The remaining proteins bound to tannins (PBT) in the pellet were extracted
186 twice with 7.5 mL SDS-solution (10 mM/Tris chloride, adjusted to pH 8.0 with 0.1 M NaOH, 10
187 g/L sodium dodecyl sulphate and 50 g/L 2-mercaptoethanol) by boiling for 60 min in a water
188 bath and cooling to room temperature in ice water. This was followed by the same centrifugation
189 and decanting procedure as above. The remaining pellet was mixed with 15 mL of HCl/butanol
190 (5:95 v/v) solution and boiled for 75 min in a water bath. Also, 1 mL of both, the unbound ET

and the PBT extract were mixed separately with 6 mL HCl/butanol solution and also boiled for 75 min in a water bath. The tubes were cooled to room temperature and the absorbance was read on a spectrophotometer (Pharmacia LKB, Uppsala, Sweden) at 550 nm. The standard was the tannin containing acetone fraction from the variety Cotswold Common, purified on a Sephadex LH20 column according to a procedure by Sivakumaran *et al.* (2004).

2.4. Statistical analyses

The statistical calculations were performed with the GLM procedure of SAS (SAS system for Windows, Version 9.1; SAS Inst. Inc., Cary, NC, USA). Dependent variables were N, BSN, NPN, AA-N, NH3-N, ET and PBT of the sainfoin varieties. Fixed effects were sainfoin variety (Cotswold Common, Teruel and Reznos; n=3), chemical treatment (no additive, acidification and PEG; n=3) and different DM stages (direct-cut and wilting; n=2). The corresponding N, BSN, ET and PBT concentrations before ensiling were included as covariates and PDIFF and the Tukey adjustment options were used for least squares means and pair wise comparisons, respectively. Simple statistics and correlation analysis of tannin and BSN were performed with Minitab 15.1 (Minitab, Inc; Pennsylvania, USA). Each chemical treatment had twelve observations (duplicates of three varieties and two DM stages) and each DM stages had 18 observations (duplicates of three varieties and three chemical treatments). Statements about the three varieties are based on one sample, each pooled at harvest. Therefore results reflect only mean varietal differences from a single location, harvest date and year. Interactions of treatments above P=0.25 were excluded from the model.

3. Results

The level of DM in the un-ensiled, direct-cut material was 225 g/kg for Cotswold Common, 173 for Reznos, 175 for Teruel and 278 g/kg for the grass/clover mixture. N values ranged from about 22 to 23 g N/kg DM for direct-cut and from 22 to 26 g N/kg DM for wilted sainfoin.

Silage pH ranged from 3.9 to 4.0 for the direct-cut and from 4.3 to 4.6 for the wilted varieties and it was not lower in the Promyr treatment. Low ammonia concentration and almost no visible signs of moulds or yeasts suggested good silage fermentation. Statistical analysis on BSN showed effects of wilting, variety and treatments and for their interactions wilting*variety, wilting*treatment, treatment*variety and treatment*variety*wilting.

3.1 Effect of variety on silage N-distribution

The Cotswold Common silage samples had higher levels of total N ($P=0.089$) compared to the Teruel and the Reznos sample and had also higher BSN levels compared to the Teruel sample ($P<0.005$). The ratio of BSN to total N was increased by wilting ($P<0.01$) and was dependent on variety ($P<0.05$). The Reznos sample had the lowest ratio of NPN to BSN, *i.e.*, the highest proportion of insoluble protein to BSN. NPN was not measured in the fresh forage because analysis of a few selected samples had shown negligible values in all varieties (Table 1).

[Table 1]

3.2 Effect of wilting on silage N-distribution

Values for N, BSN and NPN are shown in Table 1 and 2 for sainfoin and Table 3 for grass/clover. Silage N and BSN concentrations in wilted, PM treated sainfoin were 1.06 and 1.16

compared to direct cut silage ($P<0.001$) but remained unchanged in the PEG treatment. Also an interaction between wilting and PM treatment on N and BSN could be observed. N and BSN ($P<0.005$) concentrations decreased for the grass/clover silage from 26.3 to 23.9 g/kg DM and from 634 to 558 g/kg N, respectively ($P<0.005$).

[Table 2]

[Table 3]

3.3 Effect of acidification on N-fractions

Soluble proteins contributed 0.1 of the BSN content. The level of BSN was higher in sainfoin silage without additives than it was in Promyr-treated silage ($P=0.001$). The proportion of NPN in BSN ranged from 0.69 to 0.99 with a mean of 0.9 whereas the Promyr treatment had the lowest proportion of NPN for both DM stages. The low ratio of 0.69 of NPN/BSN was for the direct-cut, Promyr-treated Cotswold Common silage sample and it had also highest total N in fresh and wilted forage. Promyr treatment decreased NPN concentration ($P=0.052$) and ammonia ($P<0.05$) compared to silage without additives.

No differences in NH_3 concentration were observed due to different DM stages or sainfoin variety, nor was AA-N influenced by any treatment or variety ($P<0.09$) (Table 1).

3.4 Effect of PEG on N-fractions

Non-PEG treated sainfoin silage had approximately half the BSN proportion compared to the grass/clover control ($P<0.001$) (Table 1 and 2). PEG treatment had a strong effect on the BSN concentration in sainfoin but this was only seen in the sainfoin samples (Figure 1). It increased BSN 1.7 fold for the wilted Reznos silage sample and up to 2.6 fold for the direct-cut Reznos

263 silage sample. The highest NPN values of 619 and 585 g N/kg BSN for direct-cut and wilted
264 material respectively, were observed for the PEG-treated Cotswold Common silage. The PEG
265 treated grass/clover silage was not different from the untreated grass/clover silage and BSN
266 remained high in both. Overall, the PEG treatment had an increasing effect of BSN for tannin
267 containing plants (Table 2).
268 [Figure 1]
269
270 *3.5 Tannin composition of the sainfoin samples*
271
272 Extractable tannins in fresh material were higher in wilted than direct-cut material and also
273 higher in un-ensiled compared to the ensiled material ($P<0.001$). The ET fraction in fresh
274 material was twice as high as compared to silage. At the same time, the PBT concentration did
275 not change in absolute, but in relative terms compared to total tannins from about 0.4 in fresh to
276 about 0.70 in ensiled sainfoin. PEG treatment resulted in lowered ET concentration ($P<0.05$) and
277 PBT concentration ($P<0.001$) and higher BSN (Figure 1). Tannin concentration in Promyr treated
278 silage did not differ from silage without additives. There were only very weak or no correlations
279 in un-ensiled sainfoin between BSN and ET ($R^2=0.45$, $P=0.029$) or PBT ($R^2=-0.14$, $P=0.510$) and
280 between silage BSN and PBT + ET in un-ensiled sainfoin ($R^2=0.16$, $P=0.45$). Values from the
281 fiber bound tannin determination were excluded since centrifugation problems occurred in the
282 form of a loose pellet with floating particles, making the results unreliable.
283
284 **4. Discussion**
285

The N concentration of the sainfoin samples were generally lower than expected, probably due to harvest at flowering stage (April/May 2007) resulting in material with a relatively high stem to leaf ratio.

The results of this study show that wilting, acid treatment and variety influenced the N fractions of the silages. Cotswold Common had highest concentration of N and relatively low NPN concentration. However, PEG treatment resulted in a high NPN concentration, particularly in the Cotswold common silage, suggesting a strong protective effect of tannins in the Cotswold common silage in the non PEG treatments. In general, N concentrations of around 22 to 26 g N/kg DM for wilted and ensiled material were in between values of 12 to 30 g N/kg DM for silages and wilted material reported by Fraser (2000), Turgut and Janar (2004) and Scharenberg *et al.* (2007b). N values of different varieties of sainfoin seem to vary considerably. A comparison of 30 sainfoin varieties harvested at similar developmental stages at the National Institute for Agricultural Botany (NIAB) in Cambridge, UK in June 2008 showed N values that ranged from 16 to 29 g N/kg DM (Lorenz, unpublished).

4.1 Effects of wilting and variety on N-fractions

The increase in total N by wilting sainfoin is likely to be caused by carbon loss through respiration. However, wilting generally improves the quality of silages as it reduces silage effluents, increases the concentration of sugars in silages and in particular, inhibits enzymatic proteolysis (Henderson, 1993). In the present study, the effects of wilting on BSN and NPN were dependent on the variety. The interaction that was shown for wilting and PM treatment on N and BSN but not for wilting and PEG treatment on N and BSN could be explained by the uptake of

309 water into the wilted material when the PEG solution was sprayed on the plant material. The
310 differences in NPN, AA-N and NH_3 distribution could not coherently be explained.
311
312 *4.2 Effects of treatment and tannin concentration on N-fractions*
313
314 Acid treatment lowers the pH in the beginning of the ensiling process. This inhibits plant
315 proteolysis but is also believed to weaken the tannin protein bonds (Jones and Mangan, 1977),
316 leaving proteins more vulnerable to fermentative degradation. Low BSN and NPN values for the
317 Promyr treatment indicate that the pH was sufficiently low to at least partially inhibit enzymatic
318 breakdown. However, the pH did not drop enough to affect the protein-tannin binding which is,
319 for leaf protein (Rubisco), according to Perez-Maldonado *et al.* (1995) around pH 3.5 to 5.5.
320 In this study, the levels of the ET and PBT fractions were similar to earlier reports on wilted and
321 ensiled sainfoin (Hristov and Sandev, 1998; Scharenberg, *et al.*, 2007a; Scharenberg, *et al.*,
322 2007b). The ET seemed to contribute to approximately 0.6 of the total tannins in the direct-cut,
323 fresh material while this was reduced to a mean value of 0.3 in the ensiled material. Whether
324 these remaining tannins do not bind protein in the silage because of their chemical properties or
325 due to a physical effect remains unknown. However, the unbound ET in ensiled material, was
326 lower in the PEG treated silage ($P<0.05$) indicating more facilitated binding to PEG compared to
327 proteins.
328 The effect of PEG on BSN in silages, which was previously observed (Jones and Mangan,
329 1977), could also be seen in the sainfoin silages in the present study. The high affinity of PEG to
330 tannins may either cause an exchange of tannin bound proteins with PEG or prevent the
331 formation of protein-tannin complexes, leaving the protein susceptible to degradation.

Overall, these results indicate that interpreting the breakdown of protein in silages by tannin concentration measured by an HCl/butanol method is difficult and requires more information than merely concentrations of extractable and bound tannins. In contrast to reports dealing with effects of different tannins levels in ruminant nutrition studies (Albrecht and Muck, 1991; Min, *et al.*, 2003), there was only a very weak correlation between tannins levels and N solubility, particularly for the tannin levels in un-ensiled sainfoin and BSN in the corresponding silage. Vitti et al. (2005) concluded in a study on the nutritional effects of different legumes and their tannin concentrations that neither high nor low tannin concentrations should be attributed to detrimental or beneficial effects. A non-linear relationship between BSN and tannins analyzed by the radial diffusion method Hagerman, (1987) on sainfoin and Lotus corniculatus was observed by Hedqvist and Udén (2004).

The clear effects of PEG on silage BSN levels confirmed a protein-tannin effect, although there was only very weak correlation between tannin concentrations and N solubility in non-PEG treated silages. There were also effects of treatments (acidification, wilting and PEG) on tannin levels, which could not be reasonably explained.

Therefore it may be inevitable to investigate alternative methods of tannin analysis for elucidating the effect of tannins on proteolysis during ensiling (Waghorn and McNabb, 2003; Hedqvist, 2004) as different tannin fractions (Terrill, *et al.,* 1992), protein precipitation (Hagerman, 1987) or bindings mechanisms (Frazier, *et al.,* 2003) can be distinguished. A combination of tannin measurements was suggested by Wisdom *et al.* (1987) and McAllister *et al.* (2005) for assessing fiber digestion as tannins can also influence fiber breakdown, protein precipitation for testing biological activity, tannin molecular weight and chromophore production for quantitative chemical characterization. This could be the method of choice for small batches

of sample but is unlikely to be applicable for large number of samples as it was the case in our study.

5. Conclusions

Acidification lowered silage BSN and NPN concentrations while effects of wilting on BSN and NPN were dependent on variety. PEG treatment of sainfoin resulted in large increases in silage BSN concentrations which confirmed an effect of protein protection by tannins. However, the correlation between tannin concentration and silage N-fractions were poor in the non-PEG treatments, indicating qualitative attributes of tannins, rather than quantitative. Overall, all sainfoin varieties showed good ensiling characteristics and relatively high concentration of un-degraded protein after ensiling. This supports the belief that sainfoin is a novel forage protein resource

Acknowledgements

This work is funded by the Marie Curie Research Training Network Project "Healthyhay – The re-invention of sainfoin (EU, FP6-2005-Mobility-1, February 14, 2006). We want to thank Mrs. Elisabetta Stringano from the University of Reading for providing the sainfoin tannin standard and Mr. Samir Demdoum from CITA for providing us with sainfoin samples. Further, we also want to thank Ulf Olsson from SLU for his help with the statistical evaluation.

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Table 1. N-fractions, ET and PBT of fresh (un-ensiled), direct-cut and wilted silage with (+PM) and without Promyr (-PM)

Fresh sainfoin			Total N	BSN	NPN	AA-N	NH3-N	ET	PBT
Treatments		Variety	g/kg DM	g/kg N		g/kg BSN		g/kg DM	
Direct-cut		Cotswold Common	22.6	190	-	-	-	32.2	35.0
		Reznos	21.8	182	-	-	-	41.2	24.4
		Teruel	22.3	218	-	-	-	42.4	17.9
Wilted		Cotswold Common	25.7	284	-	-	-	50.4	33.3
		Reznos	24.2	299	-	-	-	41.2	15.9
		Teruel	21.9	259	-	-	-	57.8	22.1
Ensiled sainfoin									
Direct-cut	-PM	Cotswold Common	24.0	310	273	357	64.3	18.5	35.1
		Reznos	23.7	250	227	375	78.2	12.2	38.8
		Teruel	23.9	327	279	443	74.1	11.8	20.9
	+PM	Cotswold Common	24.7	244	167	370	61.9	17.4	32.6
		Reznos	22.3	206	201	317	30.6	13.9	26.9
		Teruel	22.3	244	216	373	32.9	9.6	16.7
Wilted	-PM	Cotswold Common	26.4	335	333	317	63.5	17.7	51.7
		Reznos	25.7	346	334	330	75.3	12.0	27.6
		Teruel	24.3	289	257	437	41.3	14.3	25.0
	+PM	Cotswold Common	26.0	326	275	277	42.9	17.1	59.4
		Reznos	26.6	309	305	376	62.1	12.2	22.3
		Teruel	23.5	280	248	337	39.9	17.4	25.0
	Mean		24.4	289	260	341	55.6	14.6	33.1
	SEM		0.32	9.24	11.10	17.50	4.60	0.70	2.80
Statistical significance						P-values:			
		Variety	>0.1	<0.05	0.052	>0.1	>0.1	<0.05	>0.1
		PM	>0.1	<0.001	<0.05	>0.1	<0.05	>0.1	>0.1
		Wilting	<0.001	<0.001	<0.001	0.090	>0.1	>0.1	<0.05
		Variety*Wilting	0.081	<0.001	<0.05	*	*	<0.05	<0.05
		PM*Wilting	*	<0.001	>0.1	*	*	*	0.082
		Variety*PM	*	*	>0.1	*	*	*	>0.1

BSN=buffer soluble N; NPN=non-protein N; AA-N=amino acid N; ET=extractable tannins; PBT=protein bound tannins.
* non significant interactions were removed from the model

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Table 2. Nitrogen fractions and tannin contents for direct-cut and wilted sainfoin silages with (+) and without (-) polyethylene glycol (PEG)

Treatments		Variety	Total N g/kg DM	BSN g/kg N	NPN	AA-N g/kg BSN	NH3-N	ET g/kg DM	PBT
Direct-cut	-PEG	Cotswold Common	24.0	310	273	375	64.3	18.5	35.1
		Reznos	23.7	250	227	443	78.2	12.2	38.8
		Teruel	23.9	327	279	395	74.1	11.8	20.9
	+PEG	Cotswold Common	22.9	652	619	329	63.5	13.8	19.1
		Reznos	23.0	602	579	219	75.3	8.2	19.5
		Teruel	22.9	536	481	362	41.3	7.4	16.3
Wilted	-PEG	Cotswold Common	26.4	335	333	329	63.5	17.7	51.7
		Reznos	25.7	346	334	219	75.3	12.0	27.6
		Teruel	24.3	289	257	362	41.3	14.3	25.0
	+PEG	Cotswold Common	23.6	593	586	380	49.6	12.6	26.9
		Reznos	24.4	586	523	418	65.9	7.5	17.0
		Teruel	22.7	581	518	328	62.7	14.4	20.6
	Mean		23.9	451	418	361	67.7	12.6	26.6
	SEM		0.24	30.4	29.5	21.0	3.7	0.8	2.2
Statistical significance						P-values:			
		Variety	<0.05	<0.05	<0.001	> 0.1	> 0.1	<0.05	<0.001
		PEG	<0.05	<0.001	<0.001	> 0.1	> 0.1	<0.05	<0.001
		Wilting	> 0.1	> 0.1	<0.05	<0.05	<0.05	> 0.1	<0.05
		Variety*Wilting	0.051	<0.05	0.07	*	*	<0.05	<0.05
		PEG*Wilting	> 0.1	> 0.1	<0.05	*	*	*	> 0.1
		Variety*PEG	> 0.1	<0.05	> 0.1	*	*	*	<0.05
		Variety*Wilting*PEG	> 0.1	<0.05	<0.05	*	*	*	<0.001

BSN=buffer soluble N; NPN=non-protein N; AA-N=amino acid N; ET=extractable tannins; PBT=protein bound tannins.

* non significant interactions were removed from the model

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Table 3. N-fractions of fresh (un-ensiled), direct-cut and wilted silage with (+PM) and without Promyr (-PM)

Grass/clover			Total N	BSN	NPN	AA-N	NH3-N
Fresh herbage			g/kg DM	g/kg N		g/kg BSN	
	Direct-cut		22.5±0.1	362±1	-	-	-
	Wilted		22.5±0.0	360±14	-	-	-
Ensiled herbage							
	Direct-cut		26.3±0.7	634±18	541±9	357±5	96±17
		+PM	24.4±0.2	538±8	450±6	326±3	24±2
		+PEG	24.4±0.1	606±3	509±3	387±2	98±3
	Wilted		23.9±0.1	558±6	476±1	316±20	53±0
		+PM	23.9±0.4	409±9	353±7	277±13	27±2
		+PEG	22.6±1.0	550±14	474±3	329±36	53±16

BSN=buffer soluble N; NPN=non-protein N; AA-N=amino acid N

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