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**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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25 **Abstract**

26

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

45

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

47

48 **1. Introduction**

49

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

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

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

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

82 Controversy exists on how to measure tannins in plants. Recent studies show that the  
83 protein-binding effects of tannins are influenced by many factors other than tannin concentration  
84 such as tannin molecular structure, their degree of polymerization, the ratio of proteins to tannins,  
85 protein structure and amino acid composition etc. (Spencer, *et al.*, 1988; Silber, *et al.*, 1998;  
86 Frazier, *et al.*, 2003; McAllister, *et al.*, 2005; Deaville, *et al.*, 2007). These questions have been  
87 reviewed by Aerts *et al.* (1999) and Mueller-Harvey (2006). Colorimetric methods like the  
88 HCl/butanol methods, originally from Porter (1992) and their modification, do by their nature not  
89 account for the qualitative characteristics listed above. They have therefore been questioned and  
90 also for the fact that colour yield is not always linear (Giner-Chavez, *et al.*, 1997a; Makkar, *et al.*,  
91 1999; Schofield, *et al.*, 2001). However, the informative value of these methods will depend on  
92 plant species and maturity, choice of standard, sample extraction, preparation method, etc..  
93 (Hagerman, 1988). Therefore, many different colorimetric methods and their modifications are  
94 still employed in attempts to predict ruminal protein metabolism (Jayanegara, *et al.*, 2009) and  
95 may, under the right conditions and within the same plant species, give reliable results and still be  
96 useful for ranking varieties within a certain species and may also allow comparison between

97 direct-cut, wilted and/or ensiled forage (Barry and Forss, 1983; Giner-Chavez, *et al.*, 1997b;  
98 Rubanza, *et al.*, 2005; MacKown, *et al.*, 2008; Rothman, *et al.*, 2009).  
99 Polyethylene glycol (PEG) has the ability to bind strongly to tannins and inhibits tannin-protein  
100 complex formation. This effect has been utilized to study if condensed tannins decrease protein  
101 degradation since PEG will limit the formation of protein-tannin complexes (Jones and Mangan,  
102 1977; Makkar, *et al.*, 2007). The objectives of this paper were to study protein metabolism during  
103 ensiling of three different sainfoin varieties. The silage treatments tested were an acidifying  
104 additive and wilting. We also attempted to test for any effects of tannin level on protein  
105 breakdown and to compare the effects of tannin level and PEG on the distribution of N in  
106 consideration of the questioned applicability of the HCl/butanol method for sainfoin silage.

107

## 108 **2. Material and Methods**

109

### 110 *2.1. Plant material*

111

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

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

124

## 125 *2.2. Ensiling procedures*

126

127 The following treatments were applied immediately before ensiling to each sample: a) PEG (100  
128 g/kg dry matter), b) Promyr (2.5 g/kg fresh matter of Promyr® MT 570) and c) no additive. PEG  
129 (Merck, Darmstadt, Germany) had a MW of 3000 Da. Promyr consisted of a solution of >750  
130 g/kg formic acid and sodium-formates in solution and <250 g/kg propionic acid (Perstorp  
131 Specialty Chemicals AB, Perstorp, Sweden). There were 12 silos of each of the 3 sainfoin  
132 cultivars and 12 silos containing grass/clover. From the 36 silos containing sainfoin, one third  
133 was treated with PEG, one third was treated with Promyr and one third was free of additives. The  
134 diluted additives were sprayed evenly over the plant material inside a plastic bag. The bag was  
135 closed and the content was thoroughly shaken in order to spread the additive evenly. Thereafter,  
136 the plant material was packed in glass silos (20 cm length and 3 cm inner diameter. After filling  
137 the silos with about 80 to 90 g, leaving approximately a 1-cm free headspace, the tubes were  
138 closed with a rubber stopper and a water lock. The silos were incubated in a dark 20°C room for  
139 60 days. The ensiled material was frozen, freeze dried and ground on a “Brabender” cutter mill to  
140 pass a 1-mm screen prior to analysis.

141

## 142 *2.3. Analytical methods*

143

### 144 *2.3.1. Dry matter, ash and N*

145 All analyses were done on both ensiled and fresh material. Dry matter of fresh samples was  
146 determined by drying at 105°C to constant weight in a forced draught oven and for ensiled  
147 material by freeze drying and multiplication with a correction factor (0.94) for remaining water  
148 and volatile losses. All N analyses were done in duplicate on the freeze dried material by the  
149 Kjeldahl procedure using a Kjeltex Analyzer unit 2400 and a 2020 Digestor (Foss, Hillrød,  
150 Denmark) with Cu as a catalyst. Buffer soluble nitrogen (BSN) was determined by extracting  
151 freeze dried samples with a borate-phosphate buffer, pH 6.75, at 39°C for 1 h according to a  
152 modified method by Licitra *et al.* (1996) as follows: 1.5 g of the freeze dried plant material was  
153 weighed into a 50-mL tube (Sarstedt, Nümbrecht, Germany) and mixed with 50 mL of the borate-  
154 phosphate buffer. The tubes were shaken and incubated for 1 h in a 39°C water bath with an  
155 additional thorough shaking every 15 min. Thereafter, the tubes were centrifuged at 3000  $\times$  g on a  
156 swing-out rotor centrifuge (G4.11, Jouan, Saint Herbain, France). Twenty mL of the supernatant  
157 was transferred to a Kjeldahl tube and analyzed for BSN. To avoid floating particles, a polyester  
158 cloth (20- $\mu$ m openings) was wrapped around the tip of the pipette and the liquid was pipetted  
159 slowly. A stepwise increase of the temperature of the Kjeltex digestion block was needed when  
160 analyzing the aqueous samples to prevent extensive foaming during digestion. Initially,  
161 temperature was slowly increased to evaporate excess water. In the last step, Cu-containing  
162 K<sub>2</sub>SO<sub>4</sub> tablets were added and the standard procedure described above for solid samples was  
163 commenced. For analysis of non-protein N (NPN), another 15 mL of the BSN extract was  
164 transferred to a 30 mL polypropylene centrifuge tube and 1.5 mL of trichloroacetic acid (TCA;  
165 200 g/L) was added and incubated for 1 h in ice water to precipitate polypeptides and proteins.  
166 After incubation, the sample was centrifuged at 27000  $\times$  g for 15 min in a spark proved Suprafuge  
167 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  $\alpha$ -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<sub>2</sub>O 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  $\times$  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

191 and the PBT extract were mixed separately with 6 mL HCl/butanol solution and also boiled for  
192 75 min in a water bath. The tubes were cooled to room temperature and the absorbance was read  
193 on a spectrophotometer (Pharmacia LKB, Uppsala, Sweden) at 550 nm. The standard was the  
194 tannin containing acetone fraction from the variety Cotswold Common, purified on a Sephadex  
195 LH20 column according to a procedure by Sivakumaran *et al.* (2004).

196

#### 197 2.4. Statistical analyses

198

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

213

### 214 3. Results

215  
216 The level of DM in the un-ensiled, direct-cut material was 225 g/kg for Cotswold Common, 173  
217 for Reznos, 175 for Teruel and 278 g/kg for the grass/clover mixture. N values ranged from about  
218 22 to 23 g N/kg DM for direct-cut and from 22 to 26 g N/kg DM for wilted sainfoin.  
219 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  
220 it was not lower in the Promyr treatment. Low ammonia concentration and almost no visible  
221 signs of moulds or yeasts suggested good silage fermentation. Statistical analysis on BSN showed  
222 effects of wilting, variety and treatments and for their interactions wilting\*variety,  
223 wilting\*treatment, treatment\*variety and treatment\*variety\*wilting.

224

### 225 3.1 *Effect of variety on silage N-distribution*

226

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

233 [Table 1]

234

### 235 3.2 *Effect of wilting on silage N-distribution*

236

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

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

243 [Table 2]

244 [Table 3]

### 245 3.3 *Effect of acidification on N-fractions*

246  
247 Soluble proteins contributed 0.1 of the BSN content. The level of BSN was higher in sainfoin  
248 silage without additives than it was in Promyr-treated silage ( $P=0.001$ ). The proportion of NPN in  
249 BSN ranged from 0.69 to 0.99 with a mean of 0.9 whereas the Promyr treatment had the lowest  
250 proportion of NPN for both DM stages. The low ratio of 0.69 of NPN/BSN was for the direct-cut,  
251 Promyr-treated Cotswold Common silage sample and it had also highest total N in fresh and  
252 wilted forage. Promyr treatment decreased NPN concentration ( $P=0.052$ ) and ammonia ( $P<0.05$ )  
253 compared to silage without additives.

254 No differences in  $\text{NH}_3$  concentration were observed due to different DM stages or sainfoin  
255 variety, nor was AA-N influenced by any treatment or variety ( $P<0.09$ ) (Table 1).

256

### 257 3.4 *Effect of PEG on N-fractions*

258  
259 Non-PEG treated sainfoin silage had approximately half the BSN proportion compared to the  
260 grass/clover control ( $P<0.001$ ) (Table 1 and 2). PEG treatment had a strong effect on the BSN  
261 concentration in sainfoin but this was only seen in the sainfoin samples (Figure 1). It increased  
262 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

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

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

300

#### 301 *4.1 Effects of wilting and variety on N-fractions*

302

303 The increase in total N by wilting sainfoin is likely to be caused by carbon loss through  
304 respiration. However, wilting generally improves the quality of silages as it reduces silage  
305 effluents, increases the concentration of sugars in silages and in particular, inhibits enzymatic  
306 proteolysis (Henderson, 1993). In the present study, the effects of wilting on BSN and NPN were  
307 dependent on the variety. The interaction that was shown for wilting and PM treatment on N and  
308 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<sub>3</sub> 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.

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

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

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

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

357

## 358 **5. Conclusions**

359

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

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374

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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	NH <sub>3</sub> -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

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

Treatments	Variety	Total N	BSN	NPN	AA-N	NH <sub>3</sub> -N	ET	PBT	
		g/kg DM	g/kg N	g/kg BSN	g/kg DM				
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	NH <sub>3</sub> -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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