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MODIFICATION OF THE PROPORTION OF EXTRACTABLE AND BOUND CONDENSED TANNINS IN BIRDSFOOT TREFOIL (*LOTUS CORNICULATUS*) AND SAINFOIN (*ONOBRYCHIS VIICIFOLIA*) DURING WILTING, ENSILING AND PELLETING PROCESSES

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Abstract: Condensed tannins (CT) in legume forages vary not only in concentration and structure, but also in the portion of soluble and protein- and fibre-bound fractions. This study aimed to assess the changes in the total CT level as well as relative abundance of the three CT fractions from fresh to wilted, ensiled or pelleted legumes like in birds foot trefoil (two cultivars) and in sainfoin (one cultivar). Each legume underwent three consecutive harvests, of which the first two were wilted. Additionally, wilted legumes were either ensiled (first harvest) or transformed into dehydrated pellets (second harvest). For each harvest, total CT and the percentage of soluble, protein- and fibre-bound CT differed ($P < 0.01$) among plants. The total CT content was similar after wilting but was lower ($P < 0.05$) after ensiling. After wilting, ensiling and pelleting the portion of soluble CT was lower in favour of protein-bound CT portion. However, time of harvest affected ($P < 0.05$) total CT and the percentage of soluble and protein-bound CT. Thus, measuring the bound-fraction should not be ignored in the determination of CT content since this fraction, together with the soluble fraction, might protect protein from ruminal degradation.

Keywords: condensed tannins, soluble fraction, bound fraction, wilting, ensiling, pelleting

Introduction

The use of legume forages in livestock farming decreased in Europe over the last two decades principally because of the low price of soyabean meal and the increasing use of corn silage (*Doyle and Topp 2004; Peyraud et al. 2009*).

However, in the last few years there is increasing interest for temperate legumes such as birds foot trefoil (*Lotus corniculatus*) and sainfoin (*Onobrychis viciifolia*). Apart from their crude protein content, the content on bioactive secondary metabolites like condensed tannins (CT) attracts great interest. Condensed tannins have been shown to improve health, production efficiency and product quality in ruminants. For instance, tanniferous legumes reduce bloat and parasitic burden and modify protein utilization through a reduction in N excreted in urine and milk thereby reducing the metabolic load (*Barry and Mcnabb 1999; Patra and Saxena 2011; Grosse Brinkhaus et al. 2016a*). Moreover, feeding CT can modify the quality of ruminant products by increasing n-3 polyunsaturated fatty acid levels and by reducing pastoral off-flavour (*Schreurs et al. 2007; Girard et al. 2015, 2016*).

Condensed tannins are a vast family of polymers composed of flavan-3-ol monomers present in different concentrations in plants. Within the same plant, CT are not equally distributed, leaves and flowers are richer in CT than stems (*Lees et al. 1993; Häring et al. 2007*). In addition to the concentration, the bioactive properties of CT play an important role (*Mueller-Harvey 2006; Frazier et al. 2010*). Bioactivityis mainly driven by the chemical structure of CT, including the mean degree of polymerization (mDP), chemical conformation (*cis*:*trans* ratio) and the ratio of procyanidin (PC) to prodelphidin (PD) monomers. Moreover, CT in the plant can be present in a soluble or insoluble form, the latter being principally bound to proteins or dietary fibres. Up to now, methods to quantify CT mainly focused on analysing the content and chemical structure of the soluble CT. Only few studies concentrated on the properties of the bound fraction of CT in relation to animal nutrition. For instance Kariuki and Norton (2008) showed that the portion bound to proteins is of interest in animal nutrition because protein can dissociate from CT and be available for digestion and absorption in the small intestine of ruminants. Furthermore, since in many livestock production systems forages are fed not only fresh but also after being conserved for months, the additional steps of the conservation process, such as drying or ensiling, may affect the content and composition of the CT and ultimately influence their bioactive properties (*Scharenberg et al. 2007a; Theodoridou et al. 2010*).Other ways of conservation like pelleting would offer a good compromise to include CT into the rations, to avoid feed wastage and to facilitate transport and storage (*Terrill et al. 2007*). However, the high temperature of this process used during pelleting might modify the bioactive properties of CT.

The present study was performed with CT-rich legumes from two different species known to differ in their CT content and in their chemical structure: two birds foot trefoil cultivars (birds foot trefoil Polom and birds foot trefoil Bull) and one sainfoin cultivar (sainfoin Perly).The study wanted to tackel the following objectives: firstly, monitor changes in the CT content from the fresh state through the wilting and ensiling or pelleting processes with special emphasis on the soluble,

protein- and fibre-bound CT fractions; secondly, assess whether the variation of the CT content and of the ratio of the three fractions with the conservation mode was similar in all three legumes; thirdly, compare these effects between harvests.

Material and methods

Forage legumes and harvest

The experiment was carried out at Agroscope Institute for Livestock Sciences, Posieux, Switzerland (latitude:46°46' N, longitude: 07°06' E; altitude: 650 m). Before sowing, fields were ploughed with a rotary harrow. Three CT-containing legumes were sown in March 2012 in fields of 7300 square meters each. The sainfoin (*Onobrychis viciifolia*, Perly cultivar; OvP) was provided by Delley Semences et Plantes (Delley semences et plantes SA, Delley, Switzerland) and the two birds foot trefoil cultivars (*Lotus corniculatus*, Bull and Polom; LcB and LcP) were provided by Cotswold Seeds (Cotswold Seeds Ltd, Gloucestershire, United Kingdom). The sowing density was 1.6 kg per are for the OvP and 300 g per are for the LcP and LcB. No mineral fertilizer was applied. To obtain three independent replicates (batches) per legume, fields were subdivided in three plots and sampling between two plots was performed at a distance of approximately 150 m (*Grosse Brinkhaus et al. 2016b*).

Legumes were harvested for the first time in July 2012 at early flowering stage for LcP and LcBand full flowering stage for OvP. The legumes were cut a second and third time after 50 days of regrowth each, in August and October 2012, respectively. In August, the bird's foot trefoil cultivars and the OvP were at full flowering and at the end of the flowering stage, respectively, whereas at the third cut all plants were at a vegetative stage. The poor yield of the third harvest hindered any further conservation trails.

Immediately after cutting, at 13:00 h, fresh samples of each legume were randomly collected in the three aforementioned plots (3 batches per field). The rest of the harvest was wilted for 24h. One day after cutting, three wilted samples were collected in the vicinity of each fresh sampling location. A fraction of each fresh and wilted sample was separated for the determination of dry matter (DM) content and the rest was stored at -20°C for further laboratory analysis.

Regarding the weather conditions during the whole experiment, from sowing to harvesting (Figure 1), rainfall was higher in June (127mm) and in September (142 mm) but with 25 mm markedly lower in March. The greatest average temperature was recorded in August with 18.8°C with temperatures ranging from 11° to 24.3°C.

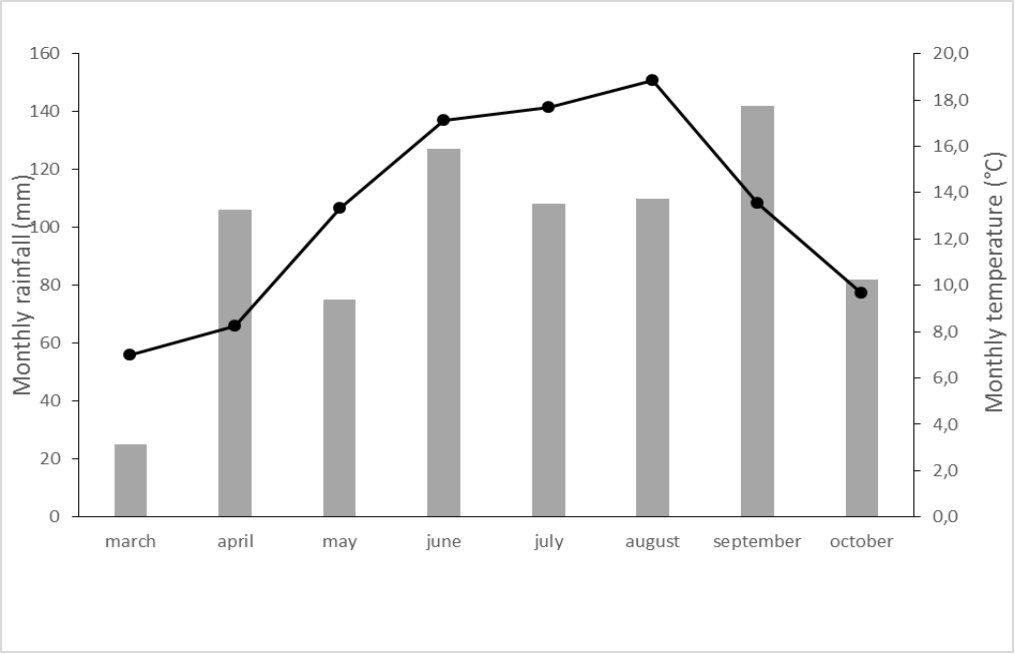

Figure 1. Monthly average temperatures (joined line) and monthly rainfall (bars) during the experimental period

Ensiling and pelleting procedures

After wilting of the first harvest, the legumes were chopped (1-2 cm) with a chaff cutter (Mex GT, Poettinger, Grieskirchen, Austria) and ensiled without additives in 1.5 L-silos. For each legume, three silos per batch were prepared. The different silos were kept at 20°C for 86 days. Afterwards, the silos were opened and pooled per batch. One subsample was used to estimate the silage DM content and a second subsample was stored at -20°C for later analysis.

The wilted forages of the second harvest were transported to a forage drying company (Trocknungsgenossenschaft des Sensebezirks, Tafers, Switzerland) for the production of dehydrated pellets. Briefly, wilted forages from the three batches of each legume were mixed together and chopped (5 to 8 cm; Neumann Würzer, Kissleg, Germany) before being dried in a rotating barrel (type 5.0, Kunz, Langnau, Switzerland). The drying process, which lasted 4 min, was a succession of heating and cooling phases repeated three times. The temperatures in the heating and cooling phases were approximately 700 and 82°C, respectively. Dried samples were finely ground (c610, Kunz, Langnau, Switzerland) and then extruded as pellets (8mm matrix, Kahl, Reinbeck, Germany).

Nutrient analysis of the samples

The DM concentration of all the samples was determined by drying at 105°C for 3 h (after a previous 24 h drying at 60°C as sample conservation means). To access chemical composition, fresh, wilted and ensiled samples were lyophilized (Christ Delta 1–24 LSC, Osterode, Germany) and ground to pass a 1-mm sieve (Brabender mill, Brabender, Duisburg, Germany). The pellets were ground to pass a 1-mm sieve. The DM content of all lyophilized and pelleted samples was quantified thermo-gravimetrically by heating at 105°C for 3 h (LECO TGA 601; Mönchengladbach, Germany). In order to determine the organic matter content, total ash content was determined by dry-ashing the samples at 550°C for 4 h. The N concentration was quantified according to the Dumas method [Association of Official Analytical Chemists (AOAC), 2000] and crude protein content was calculated (N × 6.25). The neutral (NDF) and acid detergent fibre (ADF) were analysed following standard protocols (AOAC, 1995) using an ANKOM 200⁄220 Fiber Analyzer (Ankom Technology Corporation, Fairport, NY, USA) where NDF was assayed with heat-stable amylase and sodium sulphite. Both NDF and ADF were expressed without residual ash after incineration at 500°C for 1 h. The nutrient composition of the fresh material used in this experiment is reported in the Table 1.

Determination of soluble, protein-bound and fibre-bound CT

The CT content was determined using an HCl-Butanol method based on the one previously described by *Terrill et al. (1992).* Thus, three consecutive fractions were prepared in duplicate to access soluble, protein-bound and fibre-bound CT. Briefly, soluble CT were extracted by mixing 500±1 mg of lyophilized plant material with a 20 ml acetone:water solution (70:30, v:v) containing ascorbic acid (1 gl^{-1}) and 10 ml of diethyl-ether. This mixture was then centrifuged at 25’000 g at 5°C for 15 minutes. The upper (organic) layer was then discarded and the acetone:water layer collected. The extraction and centrifugation steps were repeated once with the solid residue. Following the second centrifugation, the solid residue containing the insoluble part of CT was kept. After combining the two acetone:water fractions containing the soluble portion of the CT, acetone was removed in a rotary evaporator (Büchi Rotavapor R-205, Büchi Labortechnik AG, Flawil, Switzerland). The aqueous acetone-free fraction was added with ultrapure water type I (milli-Q) to a total volume of100 ml in a volumetric flask. The kept solid residue was mixed with 15 ml of sodium dodecylsulfate (SDS) and 2-mercapto-ethanol solution (10 respectively50 gin 1 l of water), heated for 45 min at 95°C and cooled in an ice-bath for 10 min before being centrifuged at 25’000 g for 15 min at 5°C. The aforementioned steps for the solid residue were repeated once and after each centrifugation, supernatants were collected into 50 ml volumetric flasks and filled up with the SDS:2-mercapto-ethanol solution to a total of 50 ml.

This solution contained the protein-bound CT. The remaining solid residue contained the fibre-bound CT.

The subsequent colorimetric determination was performed individually on each of the three fractions using HCl (37%):butanol (5:95; v:v) solution. Six ml of HCl:butanol (5:95; v:v) solution was added to 1 ml of extract containing soluble or protein-bound CT whereas the solid residue containing the fibre-bound CT was mixed with30 ml HCl:butanol (5:95; v:v) and 3 ml of the SDS:2-mercapto-ethanol solution. Subsequently, all samples were boiled under reflux for 1h. The reflux was carefully set so as to avoid any losses. Similarly, a blank for each fraction was prepared with water:butanol (5:95; v:v) instead of the HCl:butanol solution. After colour development, all samples were immediately cooled in an ice-bath. Then, for samples containing the fibre-bound CT, A centrifugation at 15'000 g for 15 min at 5°C was performed in order to remove the solid pellet. Finally, all samples were filtered through hydrophilic filter. Absorbance at a wavelength of 550 nm was readily measured against a blank HCl:butanol (5:95; v:v) solution using a UV/VIS Spectrometer (PerkinElmer instruments, Lambda 40).

Purification of CT for the calibrations curves

Since each legume has its characteristic CT profile (e.g. different polymer size, chemical composition and conformation, soluble/non-soluble fractions, etc.) a specific calibration curve was prepared for each legume and solvent (water or SDS:2-mercapto-ethanol solutions). Purified CT material from each legume was prepared as following: A sample (50 g) from each lyophilized fresh-legume was stirred for 40 min in a solution of acetone:water (70:30, v:v) containing ascorbic acid (1 g l^{-1}) and then vacuum filtrated. The filtrate was washed three times with 250 ml dichloromethane in a separating funnel in order to remove lipids and pigments. The aqueous phase was then evaporated and lyophilized. This lyophilized sample was dissolved in methanol:water (50:50; v:v),run through a Sephadex column (Sephadex LH-20,25-100 µm, Fluka n°84952, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) and eluted with acetone:water (70:30; v:v). The presence of CT in the different elution fractions was detected by the vanillin/HCl test (100 g l^{-1} of vanillin in 37% HCl). Finally, the eluted fractions positive to the vanillin/HCl test were combined, concentrated in a rotary evaporator and finally freeze-dried. Calibration standards of different concentrations were prepared with each purified CT material, both in water and in SDS:2-mercapto-ethanol (10:50 g l^{-1}).A total of six calibration curves were prepared, two per legume (one in water for the soluble fractions and one in SDS:2-mercapto-ethanolfor the insoluble fractions).

Statistical analysis

Except for fresh samples, data on total, soluble, protein- and fibre –bound CT levels were analysed for each harvest time separately using the procedure

MIXED of SAS (version 9.2). With the data of the first and second harvests, the plant (OvP, LcP, LCB), the forage form (fresh, wilted, silage and fresh, wilted, dehydrated pellet, respectively) and the plant × forage form interaction were used as fixed effects. As in the first harvest the batches were ensiled separately, the three batches were used as random effect in the statistical model.

Data on total, soluble, protein- and fibre–bound CT levels determined in the fresh samples of OvP, LcP and LCB were compared between the three harvests. For the mixed model the plant, the harvest time and the plant × harvest time interaction were used as fixed effects and the three batches as random effect. Least squares means were compared using the PDIFF option with the Tukey adjustment statement. All statistical tests were considered significant at $P < 0.05$.

Table 1. Nutrient composition of the fresh material at different harvest times

Plant	Harvest	Forage form	Items*				
			DM	OM	CP	NDF	ADF
Lotus corniculatus Polom	1	fresh	160.13	901.93	195.42	411.27	321.78
	2	fresh	179.60	903.32	199.16	383.73	364.81
	3	fresh	146.07	904.19	252.19	245.17	233.14
Lotus corniculatus Bull	1	fresh	153.00	902.37	194.63	429.52	336.04
	2	fresh	149.83	903.45	224.09	355.03	341.67
	3	fresh	156.43	903.58	255.04	265.77	235.65
Onobrychis viciifolia Perly	1	fresh	169.37	916.29	132.34	395.90	388.51
	2	fresh	219.43	916.75	147.98	340.98	328.20
	3	fresh	178.30	922.17	227.98	238.46	248.52

*expressed in g kg^{-1}: DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fibre; ADF, acid detergent fibre.

Results

Changes in the total CT content and the relative portion of total, soluble, protein- and fibre-bound CT in the fresh, wilted and pelleted legumes at the first harvest

The CT content and the percentage of soluble and insoluble CT from the first harvest are presented in Table 2. Regardless of forage form, total CT content was on average five times greater ($P < 0.05$) in the OvP compared with the LcP and LcB. With respect to the different fractions, relative differences ($P < 0.01$) between plants in the percentage of the 3 fractions can be observed. The average relative content of the soluble and protein–bound fractions in fresh, wilted and ensiled LcP was similar counting for 45 and 43% of the CT respectively, whereas relative content of the fibre bound fraction was12% lower ($P < 0.05$). By contrast, in LcB the average relative content of the soluble CT fraction was with 70% the most

abundant fraction, whereas the relative content of the protein- and fibre-bound fractions was 18 and 12% lower ($P < 0.05$), respectively. In fresh, wilted and ensiled OvP, almost two- and one-third of the CT were present in the soluble and protein-bound fractions, respectively, whereas the level of fibre-bound CT was only 9%. Regardless of the forage form, when comparing between legumes the average percentage of soluble CT was the greatest ($P < 0.05$) in LcB, followed by OvP and the lowest in LcP. Contrarily, percentage of protein-bound CT was lower ($P < 0.05$) in LcB, followed OvP and LcP. The percentage of fibre-bound CT was greater ($P < 0.05$) in the 2 birds' foot trefoil cultivars than in OvP.

With respect to the conservation mode, the average CT content of the 3 legumes decreased by 27% between fresh and silage samples. However, during the ensiling process, the relative abundance of soluble CT progressively declined ($P< 0.05$) reaching a difference of 19% between fresh and ensiled samples. Concomitantly, the protein-bound CT fraction increased ($P < 0.05$) by 17% from fresh to silage samples. The percentage of the fibre-bound CT fraction increased ($P < 0.05$) from fresh to the wilted samples but afterwards in the silage levels were comparable to the fresh samples.

Table 2. Changes in the total condensed tannins (CT) content and the relative portion of soluble (S-CT), protein- (P-CT) and fibre-bound CT (F-CT) in fresh, wilted and ensiled *Lotus corniculatus Polom*, *Lotus corniculatus Bull*and *Onobrychis viciifolia Perly*at the first harvest[1]

	Total CT (g kg^{-1} DM)	Percentage of		
		S-CT	P-CT	F-CT
Lotus corniculatus Polom				
fresh	23.1c	51de	38ab	11abc
wilted	24.4c	41f	43ab	16a
silage	23.1c	44ef	46ab	10bc
Lotus corniculatus Bull				
fresh	34.3c	75ab	13d	12ab
wilted	33.2c	68b	17d	15ab
silage	34.8c	66bc	23cd	11abc
Onobrychis viciifolia Perly				
fresh	174.1a	79a	15d	6c
wilted	155.5a	56cd	33bc	11abc
silage	111.6b	38f	51a	11abc
SEM[2]	8.62	2.0	2.5	1.2
P-values				
plant	<0.001	<0.001	<0.001	0.002
forage form	0.027	<0.001	<0.001	<0.001
plant × forage form	0.011	<0.001	<0.001	0.033

[1]Within a column, means not sharing lowercased letters differ significantly at the $P < 0.05$ level.

[2]SEM = standard error of plant × forage form mean

A plant × forage form interaction ($P < 0.05$) existed for total CT content as well as for the percentage of soluble, protein-bound and fibre-bound CT. Regardless of the forage form, total CT content did not change in the 2 birds' foot cultivars. By contrast, total CT content was lower in the OvP silage compared with the fresh and wilted OvP (interaction plant × forage form; $P < 0.05$). The percentage of soluble CT decreased and the percentage of protein-bound CT increased in the OvP, whereas in fresh, wilted and silage samples of the 2 birds foot cultivars the contents did not change (plant × forage form; $P < 0.05$).

Changes in the total CT content and the relative portion of total, soluble, protein- and fibre-bound CT in the fresh, wilted and pelleted legumes at the second harvest

Similar to the first harvest, in the second harvest average total CT content of OvP was greater ($P < 0.05$) compared with the 2 birds foot trefoil cultivars (Table 3). Regardless of the forage form, the percentage of soluble CT decreased ($P < 0.05$) from OvP to LcB to LcP. The percentage of protein-bound CT was greater ($P < 0.05$) in LcP compared with LcB and OvP while the percentage of fibre-bound CT was greater ($P < 0.05$) for the 2 birds' foot trefoil compared with the OvP.

Table 3. Changes in the total condensed tannins (CT) content and the relative portion of soluble (S-CT), protein- (P-CT) and fibre-bound CT (F-CT) in fresh, wilted and pelleted *Lotus corniculatus Polom, Lotus corniculatus Bull* and *Onobrychis viciifolia Perly* at the second harvest[a]

	Total CT (g kg^{-1} DM)	Percentage of		
		S-CT	P-CT	F-CT
Lotus corniculatus Polom				
fresh	31.5	59d	30c	11
wilted	27.1	49e	39b	12
pellets	24.8	34f	50a	16
Lotus corniculatus Bull				
fresh	39.3	73b	17e	10
wilted	41.0	70bc	20de	10
pellets	36.4	65cd	22de	13
Onobrychis viciifolia Perly				
fresh	207.0	79a	15e	6
wilted	166.3	69bc	22d	9
pellets	140.4	69bc	21de	10
SEM[b]	16.30	1.7	1.8	1.6
P-values				
plant	<0.001	<0.001	<0.001	0.002
forage form	0.069	<0.001	<0.001	0.007
plant × forage form	0.104	<0.001	0.001	0.677

[a]Within a column, means not sharing lowercased letters differ significantly at the $P < 0.05$ level.
[b]SEM = standard error of plant × forage form mean

Regardless of the legumes, dehydrated pellets had a greater ($P < 0.05$) percentage of fibre-bound CT than the fresh and wilted samples. The average percentage of soluble CT was 20% lower ($P < 0.05$) and that of the protein-bound CT portion 50% greater ($P < 0.05$) in dehydrated pellets compared to the fresh samples. However, a plant × mode of conservation interaction existed ($P < 0.001$) for the percentage of both soluble and protein-bound CT (Figure 3). This interaction was mainly caused by the steady decrease in the portion of the soluble CT fraction and an increase in the protein-bound fraction from fresh to wilted and pelleted LcP samples. Only minimal changes in the relative portions of the 3 fractions occurred in the LcB and OvP.

Changes in the total CT content and the relative portion of total, soluble, protein- and fibre-bound CT in the fresh legumes depending on the time of harvest

In all three cuts, the total CT content and the relative portions of soluble, protein- and fibre-bound CT fractions in fresh samples differed ($P < 0.001$) among legumes (Table 4). Also when the third harvest was included, total CT content of the OvP was still 5 to 6 times greater ($P < 0.05$) compared with the 2 birds foot trefoil cultivars. Both, OvP and LcB had a greater ($P < 0.05$) relative portion of soluble and a lower ($P < 0.05$) portion of protein-bound CT than the LcP. The portion of fibre-bound CT was greater for the 2 birds' foot trefoil cultivars compared with the OvP.

Table 4. Changes from different harvest times in the total condensed tannins (CT)content and in the relative portion of soluble (S-CT), protein- (P-CT) and fibre-bound CT (F-CT) in fresh Lotus corniculatus Polom, Lotus corniculatus Bull and Onobrychis viciifolia Perly(OvP)[a]

		Total CT	Percentage of		
Plant	harvest	(g kg^{-1} DM)	S-CT	P-CT	F-CT
Lotus corniculatus Polom	1	23.1	51bc	38	11ab
	2	31.5	59b	30	11ab
	3	19.4	47c	41	12a
Lotus corniculatus Bull	1	34.3	75a	13	12a
	2	39.3	73a	17	10abc
	3	37.4	75a	17	8bcd
Onobrychis viciifolia Perly	1	174.1	79a	15	6d
	2	207.0	79a	15	6d
	3	164.5	75a	18	7cd
SEM[b]		8.33	2.0	2.3	0.8
P-values					
plant		<0.001	<0.001	<0.001	<0.001
harvest		0.031	0.020	0.031	0.220
plant × harvest		0.194	0.030	0.086?	0.003

[a]Within a column, means not sharing lowercased letters differ significantly at the $P < 0.05$ level.
[b]SEM = standard error of plant × harvest mean

The total CT content and on average the percentage of soluble CT were greater ($P < 0.05$) in the second than the third harvest with intermediate values for the first harvest. However, the differences in the portion of soluble CT was mainly observed in the LcP, but not in LcB and OvP (plant × harvest interaction: $P < 0.05$). The percentage of protein-bound CT was on average lower ($P < 0.05$) in the second compared with the third harvest with intermediate values for the first harvest. Except for LcB, where the portion of fibre-bound CT was greater in the first compared to the third harvest, the changes in the relative portion of the fibre-bound fraction in LcP and OvP were minimal (plant × harvest interaction: $P < 0.003$)

Discussion

Effect of the plant species and plant cultivar on CT content the percentage of the CT fractions

The total CT content differed primarily between plant species and only numerically between the two birds' foot cultivars (LcB >LcP). By contrast, some authors demonstrated that the CT content of birds foot trefoil as well as sainfoin cultivars differ (*Acuña et al. 2008; Azuhnwi et al. 2011*). However, the current observations are in line with results of *Scharenberg et al. (2007a)* who reported greater CT content in sainfoin than in birds' foot trefoil. The CT content of the LcP and LcB are similar to previously reported values of other cultivars using the same method *(Terrill et al. 1992; Scharenberg et al. 2007a*). The CT contents determined in the OvP were on average two times greater compared to earlier experiments from our group in which the total CT content of sainfoin accessions (cultivars such as Visnovskyor Perly, ecotypes or landraces) ranged from 50 to 100 g kg^{-1} DM (*Scharenberget al. 2007a 2007b; Azuhnwi et al. 2011*). However, others reported CT contents of 120 g kg^{-1} DM in the variety Nova of sainfoin (Li et al. 2014). Besides the cultivars, the CT content is depending on many agronomic and environmental factors such as the photoperiod, the temperature and the type of soil (*Theodoridou et al. 2011a*). The unexpected great concentrations for the OvP compared to previous studies could also result from the calibration standard used to quantify the CT content. In the present study, a standard of each plant and each cultivar was purified from the fresh material from the first harvest. Thus, 3 calibration standards were used, whereas in the study of *Azuhnwi et al. (2011)* only one calibration standard from sainfoin was used for all the cultivars and accessions. By using qualitative rather than quantitative analytical methods, such as thiolysis *(Gea et al. 2011),* it revealed the heterogenic chemical structure of CT. For instance, it has been shown that sainfoin contains more PD than PC while birds' foot trefoil contains more PC than PD (*Foo et al. 1996; Gea et al. 2011).* Similarly, the mDP can differ according to the plant. For instance, the mDP in

sainfoin is usually higher than the one in birds foot trefoil with reported mDP values up to 70 and 20 for sainfoin and birds foot trefoil respectively (*Meagher et al. 2004; Gea et al.2011*).An additional indication of the complexity of the CT is the findings that the percentage of the soluble, protein- and fibre-bound CT fraction differs between plants and the cultivars. In the OvP and LcB the soluble CT fraction represented >58% of total CT whereas this portion was only up to 48% in the LcP.

Effect of the harvest on CT content and the percentage of the CT fractions

The content of extractable CT increases only numerically in the present study between the first and the second harvest. The review of *Wang et al. (2015)* reported that this content increases usually after regrowth. The reason for this increase is not fully elucidated. Various possible explanations have been proposed. Firstly, the increase in CT content could be linked to higher and drier temperature and a longer photoperiod in the second compared to the first growth period which occurs usually in early summer *(Lascano et al. 2001; Wang et al 2008; Theodoridou et al. 2011a; Li et al. 2014).* Secondly, the increase in the plant biomass and the concomitant increase in the portion of leaves after regrowth (*Häring et al., 2007*). Finally, producing more CT could also be a defence response against herbivores and plant pathogens to dissuade them from eating the plant.

An interesting finding of the present study was the fact that harvest time point affects qualitatively the CT content of fresh legumes, the main differences being observed between the second and the third harvest. Previous studies already reported qualitative change of CT like PC:PD and *cis*:*trans* ratio between two successive harvests. *Azuhnwi et al. (2013)* found a general tendency to lower PC portion and *cis* configuration between the primary growth and regrowth. Ultimately, changes in the polymer composition can modify the properties of CT to interact with proteins (*Sarni-Manchado et al. 1999; Frazier et al. 2010*).

However, as reported by *Theodoridou et al. (2011b),* the differences observed might not solely be the result of time of harvest but probably more due to differences in the phenological stage and thus plant maturity between the harvests. In the current experiment, the third harvest was carried out at the vegetative, thus less mature stage of the three plants, whereas the first and second harvest were performed at an intermediate and very advanced stage of maturity, respectively. Thus, if the maturity of the plant is considered independently of the time of harvest, the CT content is progressively increasing from the least to the more advanced stage of maturity, especially for the LcB and the OvP. This is in line with results obtained by *Theodoridou et al. (2011a)* and could be explained by the fact that at a more mature stage, legumes are developping the flowers which are rich in CT. In addition, it seems that with advanced maturity, the portion of protein-bound CT is decreasing in favor of an increase in soluble CT, particularly for the LcP. In contradiction to *Wang et al. (2015), Theodoridou et al. (2011b)* observed a

decrease in the mDP from the first (end of flowering) to the second vegetation cycle (start of flowering) indicating that with increasing plant maturity CT polymers become shorter. Shorter polymers have a reduced affinity to bind protein as fewer numbers of active sites on the CT molecule are available (*de Freitas and Mateus 2002*). Moreover, *Theodoridou et al. (2011a)* already showed that nitrogen concentration is decreasing with plant maturity because nitrogen is mainly in the leaves (Borreani et al. 2003) and the leaf-to-whole-plant ratio is decreasing with plant maturity. Consequently, a reduced affinity to bind protein associated to a decrease in nitrogen content in the plant withadvanced maturity could explain why the protein-bound CT are decreasing in the present study.

Effect of wilting, ensiling and pelleting on CT content and the percentage of the CT fractions

Forage conservation methods not only alter the nutrient composition (*Wyss, 2013*) but also the CT content of legumes (*Lorenz et al. 2010*). Although wilting had no clear effect on the total CT content, the level was lower after ensiling and tended to be even lower after pelleting. The reason for the decrease in the CT content from fresh to ensiled or pelleted legumes might be due to oxidative processes caused by fermentation during ensiling and by high processing temperature during the pelleting. The HCl butanol method used in this study did not allow to determine the extent of oxidized CT. However, from fresh to silage and pellets a 36 and 33% decrease in the total CT content in OvP but not LcB or LcB were observed in the present study. One possible reason for this finding could be related to the nature of the CT, such as a greater PD content in OvP compared to birds' foot trefoil which could be oxidized more easily than PC (*Foo and Porter 1980*).

The current results are in line with other studies who found that compared to fresh forage, hay of *Sericea lespedeza* or sainfoin contained less extractable CT (*Terrill et al. 1990; Aufrère et al. 2008*). Nevertheless, the new approach here compared to previous studies was to monitor the changes of each CT fraction during the conservation of the forage independently of the total CT content. The insoluble portion of CT is interesting from an animal nutrition perspective as it has been shown that protein-bound CT can dissociate in the small intestine of ruminants and makes protein available for digestion (*Kariuki and Norton, 2008*). In addition, the study of *Grosse Brinkhaus et al. (2016b)* showed that an OvP silage, with 60% of protein-bound CT, supplied more duodenally utilisable crude protein relative to total crude protein than other legumes. The increase in protein- and fibre-bound CT fractions confirms results obtained on sainfoin by *Scharenberget al. (2007b)* who compared hay and silage and by *Terrill et al. (2007)* who showed that pellets of *Sericea lespedeza* contained mainly protein-bound CT. In the present study, the pelleting process has the same effect as hay making or ensiling. During the whole ensiling and pelleting process, the portion of soluble CT continuously

decreases from fresh to wilted and to silage or pellets. This decrease is accompanied by a concomitant increase in the protein-bound CT fraction and regarding pelleting process, an increase in the fibre-bound fraction. *Minnee et al. (2002)* hypothesized that during conservation, the plant cells are damaged allowing the release of previously sequestered soluble CT from the vacuole into the cytosol and form complexes with proteins and fibres. This course of possible events would be in line with the present findings.

Conclusion

The present study demonstrated that the plant species and the different modes of conservation can affect quantitatively as well as qualitatively total CT content as well as the relative portion of the three CT fractions of forages. Total CT content can be characterized in terms of chemical structure with the development of methods such as in situ thiolysis and the soluble part of the CT can be easily extracted with acetone and water and characterized as well by thiolysis or LC-MS/MS. For animal nutritionists, the interest in the soluble part of the CT comes from their ability to affect ruminal fermentation by preventing protein degradation via complex building and thus protecting both dietary and endogenous proteins and/or indirectly by affecting microbial activity. However, the present results showed that the insoluble portion of CT is with over 50% of the total CT the main fraction in silage and dehydrated pellets. In the case of conserved forages, it would be interesting to get a better understanding of the relevance of the bound CT fractions in ruminant nutrition. Hence, the question arises whether these plant protein which have been protected from ruminal degradation because they were bound to CT can dissociate from CT in the small intestine and be available for absorption. Thus, in subsequent studies a better characterization of the chemical properties of these bound portions needs to be envisaged.

Modifikacija proporcije ekstrabilnih i vezanih kondenzovanih tanina u žutom zvezdanu (*Lotus corniculatus*) i esperzeti (*Onobrichis viicifolia*) tokom procesa sušenja, siliranja i peletiranja

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Rezime

Kondenzovani tanini (CT) u leguminozama se razlikuju ne samo u koncentraciji i strukturi, već i u delu rastvorljivih frakcija koje su vezane za proteine i vlakna. Ova studija je imala za cilj da proceni promene u ukupnom nivou CT, kao i relativno obilje tri CT frakcije od svežih do osušenih, siliranih ili peletiranih mahunarki kao što su žuti zvezdan (dve sorte) i esperzeta (jedna sorta). Svaka vrsta je imala tri uzastopne žetvama, od kojih su prve dve sušene. Pored toga, osušene mahunarke su ili silirane (prva žetva) ili transformisane u dehidrirane pelete (druga žetva). Za svaku žetvu, ukupni CT i procenat rastvorljivog CT-a vezanog za protein odnosno vlakna, razlikovali su se ($P < 0,01$) među biljkama. Ukupan CT sadržaj je bio sličan nakon sušenja, ali je bio manji ($P<0,05$) nakon siliranja. Posle sušenja, siliranja i peletiranja, deo rastvorljivog CT-a bio je niži u korist CT-a vezanog za protein. Međutim, vreme žetve je uticalo ($P <0,05$) na ukupni CT i procenat rastvorljivog i CT vezanog za protein. Prema tome, merenje vezane frakcije ne treba zanemariti u određivanju sadržaja CT-a, jer ova frakcija, zajedno sa rastvorljivom frakcijom, može zaštititi protein od degradacije u rumenu.

Ključne reči: kondenzovani tanini, rastvorljiva frakcija, vezana frakcija, sušenje, siliranje, peletiranje

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