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Protein fractions as influenced by cultivars, stage of maturity and cutting dates in alfalfa (*Medicago sativa* L.)

Jordan Marković[1], Mirjana Petrović[1], Dragan Terzić[1], Tanja Vasić[1],
Ivica Kostić[1], Ratibor Štrbanović[2], Goran Grubić[3]

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ABSTRACT

This study was undertaken to determine the relationship between CNCPS (Cornell Net Carbohydrate and Protein System) protein fractions and *in vitro* RUP (Rumen Undegradable Protein) concentration and the variability of protein fractions among alfalfa cultivars grown in Serbia. Two cultivars of alfalfa (*Medicago sativa* L.) - Serbian *cv* K 28 and American *cv* G + 13R + CZ were sampled at three stages of maturity. Comparing the two cultivars of alfalfa (K 28 *vs*. G + 13R + CZ) means, there were significant differences in all protein fractions. Two investigated alfalfa cultivars differed significantly ($p < 0.01$) in RUP content, *cv* G + 13R + CZ was higher in RUP than *cv* K 28. Stage of maturity had an effect on proportions of the protein fractions. From a nutritional and breeding point of view, cultivar such as G + 13R + CZ is desirable because it combine higher CP (Crude protein) values with lower protein degradability than *cv* K 28.

Key words: Alfalfa, Cultivars, Cut, Protein degradability, Protein fractions.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is the most important forage legume in the temperate climate (Karayilanli and Ayhan, 2016; Štrbanović *et al.,* 2015) becouse of high yield and high nutrient levels (Yu *et al*., 2003; Karayilanli and Ayhan, 2017). It is grown on over 30 million hectares globally, and on about 200,000 ha in Serbia (Djukić, 2005). It is an important source of protein for ruminants, but its protein is often poorly used because it is extensively degraded during ruminal fermentation (Yu *et al*., 2003). This degradation may be the most limiting factor of high-quality forage legumes.

Significant genetic variation has been reported in alfalfa for ruminal *in vitro* protein degradability (Guines *et al*., 2003; Tremblay *et al*., 2000). Botanical traits, nutritive value and CP (crude protein) fractions of alfalfa are influenced by cultivar, stage of maturity (SM) (Yu *et al*., 2003; Coblentz *et al*., 2008) and climate condition (Lamb *et al*., 2003).

Accurate predictions of different protein fractions is an essential requirements for improving the nutrient use efficiency of ruminants. These fractions influence the amount of CP degraded in the rumen and escaping to the lower digestive tract (Lanzas *et al*., 2007; Jonker *et al*., 2011). The CNCPS (Cornell Net Carbohydrate and Protein System) is a methematical model designed to evaluate the nutrient requirements and supply of cattle over a wide range of environmental, dietary, management and production situations. Many current nutritional models for ruminants require knowledge of the concentrations of rumen degradable protein (RDP) and rumen undegradable protein (RUP) within forages (Coblentz *et al*., 2008).

The hypothesis of the present study is that protein degradation may be predicted by the separation of total forage CP into solubility fractions. The objective of the present study were: to compare protein solubility fractions across alfalfa cultivars, stages of maturity and harvesting forages from the first to the fourth cut.

[1]Institute for Forage Crops Kruševac, 00 381 37 251 Globoder, Kruševac, R Serbia.
[2]Institute for Plant Protection and Environment, Teodora Drajzera 9, 00 381 11 000 Belgrade, R Serbia.
[3]Faculty of Agriculture, University of Belgrade, Nemanjina 6, 00 381 11 080 Zemun-Belgrade, R Serbia.

Corresponding Author: Jordan Marković, Institute for Forage Crops Kruševac, 00 381 37 251 Globoder, Kruševac, R Serbia. Email: jordan.markovic@ikbks.com

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MATERIALS AND METHODS

This experiment was carried out in the experimental field of Institute for Forage Crops in Kruševac (43°34′58″N, 21°19′35″E). The study area was situated at altitude of 166 m above sea level in Central Serbia. Two cultivars of alfalfa (*Medicago sativa* L.) - Serbian *cv* K 28 selected at Institute for forage crops, Kruševac and American *cv* G + 13R + CZ selected at UC Davis Plant Breeding Center, University of California were sampled at three stages of maturity, corresponding to the cutting dates shown in Table 1. Plants from a pure stand were cut manually with scissors about 5 to 7 cm above the soil surface. Samples were dried to constant weight at 65°C for 48 h and dried samples were ground through a screen size of 1 mm. All analysis were done in duplicate and component concentrations were corrected to a 100°C dry matter basis.

Cutting dates and estimated stages of maturity of forages

Cutting was taken up at three maturity stages and four different periodic intervals of time during the crop cycle. Maturity stages include *viz.*, full bud (FB), early bloom (EBL) with 10-15% flowering and Mid bloom (MBL) with 50-60% flowering.

Assessing leaf to total stem weight ratio (%) at three different maturity stages

To assess leaf and stem proportion, each plot was sampled at three maturity stages *viz.*, full bud (FB), early bloom (EBL) and mid bloom (MBL). These subsamples were dried at 65°C in forced-air oven, weighed and then stems were separated from the leaves which constituted the sub samples at each maturity stage. The weight of leaf and stem portions were estimated individually for each sample (Table 2).

Estimation of fractional rate of degradation (Kd) of protein sub fractions

Fractionation of CP in alfalfa forage was conducted according to the CNCPS (Sniffen *et al*., 1992). According to this system, CP is partitioned into three fractions: fraction A is nonprotein nitrogen (NPN × 6.25); fraction B is true protein, and fraction C is unavailable protein. Fraction B is further devided into three subfractions (B_1, B_2 and B_3) that are believed to have different rates of ruminal degradation. Fraction C is the protein that is insoluble in acid detergent (acid detergent-insoluble protein, ADICP).

Crude protein was determined as Kjeldahl N × 6.25 (AOAC, 1990). Precipitated true protein (TP), buffer-insoluble protein (IP), neutral detergent-insoluble protein (NDICP) and acid detergent-insoluble protein (ADICP) were analyzed as described by Licitra *et al.* (1996).

Fraction A was calculated as the difference between the total CP and precipitated true protein. True protein was determined by Kjeldahl analysis of the residue resulting after precipitation with trichloracetic acid (10% w/v in water) followed by filtration. Fraction B_1 was estimated as true protein minus buffer-insoluble protein, fraction B_2 as buffer-insoluble protein minus NDICP, and fraction B_3 by subtracting the ADICP (fraction C) from the NDICP according to Fox *et al.* (2004).

The CNCPS (Cornell Net Carbohydrate and protein System) is a methematical model designed to evaluate the nutrient requirements and supply of cattle over a wide range of environmental, dietary, management and production situations. Rumen-degradable CP (RDP) was calculated based on CNCPS subfractions using fractional rate of degradation (Kd) values given for legume pasture (Grabber, 2009). Rumen degradable protein (RDP) was calculated as follows:

$$RDP = \sum CP \text{ sub-fractions} \times Kd / (Kd + Kp)$$

Where

Kp is fractional rate of passage which is assumed to be 0.045 h^{-1}. Fractional degradation rates of CP sub-fractions adapted from legume pasture values reported in the CNCPS v_6.1 feed library (www.cncps.cornell.edu). Rumen-undegradable CP (RUP) was calculated by subtracting RDP from total CP (Table 3).

Experiment was established as a randomized complete block design in three replications, with factorial arrangements of three main factors (2 alfalfa cultivars × 3 stage of maturity × 4 cuts). Data were used to test the effects of stage of maturity, cuts and their interactions on protein fractions, RDP and RUP for each alfalfa cultivar separately. The data were processed by the analysis of variance in a randomized block design (ANOVA, Stat. Soft. STATISTICA 6). The significance of differences between arithmetic means was found out by Tukey test ($p<0.01$). Correlations between variates were computed on cultivar means and principal component analysis (PCA) was performed using STATISTICA 6.

Results and Discussion

Mean performance of the two alfalfa cultivars for crude protein fractions at different maturitz dates and different cutting intervals

Comparison of the mean performance indicated significant differences in all protein fractions among the two cultivars. The results indicate that alfalfa *cv* G + 13R + CZ was higher in CP content and rapidly degradable PA fraction. Among the sub fractions of true protein, K 28 registered higher mean of PB_1 and the slowly degradable PB_3 fraction while the cultivar G+13R+CZ had higher mean PB_2, an intermediately degraded protein fraction associated with the cell wall. Significant differences could be observed in respect of

Table 1: Cutting dates and estimated stages of maturity of forages.

Maturity stage cut	FB- Full bud	EBL- Early bloom 10-15% of flowering	MBL- Mid 50-60% of flowering
I	04 May (60)*	21 May (77)*	29 May (85)*
II	08 June (35)*	15 June (42)*	21 June (48)*
III	06 July (23)*	13 July (30)*	18 July (35)*
IV	08 August (26)*	16 August (34)*	21 August (39)*

*Number in the parentheses indicate the number of days of the growing cycle.

Table 2: The leaf to weight ratio, %.

SM	FB		EBL		MBL	
cv	K 28	G+13R+CZ	K 28	G+13R+CZ	K 28	G+13R+CZ
I cut	39.5[c]	41.1[b]	38.1[c]	40.7[c]	36.3[c]	38.5[b]
II cut	38.3[c]	41.1[b]	35.8[d]	39.7[c]	33.1[d]	32.5[c]
III cut	45.7[b]	48.5[a]	44.2[b]	43.2[b]	39.7[b]	40.3[a]
IV cut	48.9[a]	49.9[a]	46.4[a]	45.2[a]	43.3[a]	41.3[a]

SM- Stage of maturity; FB- Full bud; EBL- Early bloom; MBL- Mid bloom; Different letters denote significantly different means (p<0.01).

Table 3: Calculation and fractional rate of degradation (Kd) of protein sub-fractions.

Protein fractions	Calculations	Kd (h^{-1})
PA	NPN (% CP)	2.00
PB_1	SolCP (% CP) - PA	0.20
PB_2	100 - (PA + PB_1 + PB_3 + PC)	0.15
PB_3	NDICP (% CP) - PC	0.08
PC	ADICP (% CP)	-

PA- Non protein nitrogen, NPN x 6.25; PB_1- Protein which is soluble in phosphate-borate buffer and are rapidly degraded in the rumen; PB_2- Protein which is insoluble in the buffer but is soluble in neutral detergent solution; PB_3- Insoluble in the buffer and in neutral detergent but is soluble in acid detergent solution; PC- Protein that is insoluble in acid detergent solution; SolCP- Protein which is soluble in phosphate-borate buffer; NDICP- Neutral detergent insoluble crude protein; ADICP- Acid detergent insoluble crude protein.

rumen degradable protein (RDP) and rumen undegradable protein (RUP) content among the two cultivars under investigation. K 28 cultivar recorded a higher mean of RDP while the other cultivar G+13R+CZ registered high mean RUP content (Table 4).

Composition of the crude protein (CP) fractions of the two alfalfa cultivars cut at different stages of maturity and cutting intervals

Stage of maturity had a profound effect on proportions of the protein fractions. The results indicated that as maturity of the cultivar advanced, crude protein and slowly degradable fraction (PB_3) decreased (p<0.01). The mean value of PB_3 fraction did not differ significantly between EBL and MB stage. However, the content of the rapidly degradable protein (PB_1) and undegradable protein fraction increased (p<0.01). A highly rapidly degradable PA fraction increased from FB stage to EBL and after that content of this fraction decreased (p<0.01), whereas the intermediately degradable PB_2 fraction decreased from FB stage to the EBL, and after that content of this protein fraction increased (p<0.01) with maturation. With regard to RUP content, increasing trend was observed at C_1 during the crop cycle. However, RDP decreased from early bloom stage (EBL) to Mid-bloom (MBL) and continued to exhibit a decreasing trend as the crop advanced to mid bloom stage.

Alfalfa cultivars differed in CP and protein fractions content (p<0.01) between cuts. The highest content of CP, PA and PB_3 was found in cut IV and the lowest in cut I (p<0.01). The concentration of PB_2 in cuts II and III was similar (p>0.01), and significantly differed in comparison with cuts I and IV. The level of PB_1 in the alfalfa samples of cut III was the highest (p<0.01) and PC fraction was the highest in the alfalfa samples of cut I, but the lowest in the samples of cut III (p<0.01). The highest content of alfalfa RUP was in cut I and the lowest in cut III, with significant differences between cuts (p<0.01; Table 4).

Correlation analysis between different fractions of proteins in alfalfa cultivars

Fraction PA was negatively correlated with PB_2 (r = -0.747) and RUP (r= -0.592) but positively correlated with RDP (r= 0.592). PC fraction was negatively correlated with RDP (r= -0.650) and crude protein was negatively correlated with PC fraction (r= -0.615). The true protein fraction PB_2 registered a negative and non significant association with RDP (r= -0.338), PC (r= -0.219) and PB_3 (r= -0.018), while the association between PB_2 and RUP was positive and non-significant (r= 0.338) (Table 5).

Principal component analysis for protein fractions at four different cuts in the two alfalfa cultivars

The PC scores for the first axis (40% of the total variation) defined a contrast RUP, PB_1, PB_2 and PC versus CP, PA

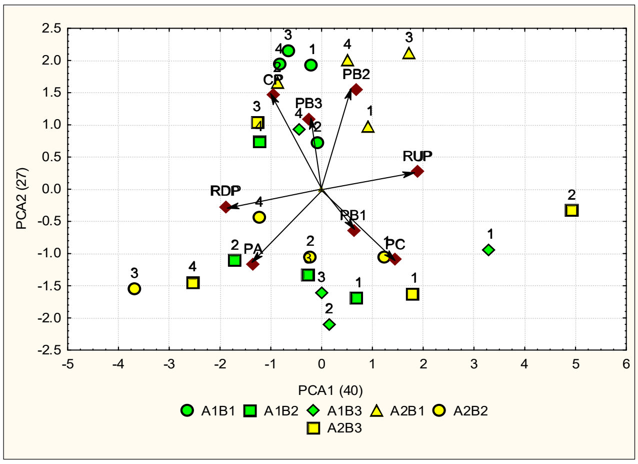

Fig 1: PCA diagram of the loadings and scores of the first principal components of the four harvested alfalfa cultivars K 28 and G + 13R + CZ.

Table 4: Protein fraction of alfalfa depending on cultivars, stage of growth and different cuts.

		CP, $g\ kg^{-1}$	DM	PA, $g\ kg^{-1}$	CP	PB_1, $g\ kg^{-1}$	CP	PB_2, $g\ kg^{-1}$	CP	PB_3, $g\ kg^{-1}$	CP	PC, $g\ kg^{-1}$	CP	RDP,g kg^{-1}	CP	RUP,g kg	CP
		a_1	a_2	a_1	a_2	a_1	a_2	a_1	a_2	a_1	a_2	a_1	a_2	a_1	a_2	a_1	a_2
c_1	b_1	199.8	196.4	346.4	348.8	0.0	0.0	549.5	561.8	48.2	8.5	55.9	81.0	792.0	778.5	208.0	221.5
	b_2	160.8	177.5	429.2	421.6	68.7	33.5	386.1	410.6	22.2	26.5	93.7	107.8	786.8	772.3	213.2	227.7
	b_3	150.2	169.6	364.3	406.6	29.5	72.7	485.4	384.4	10.4	23.0	110.3	113.2	739.5	767.2	260.5	232.8
c_2	b_1	203.5	213.4	410.8	341.5	0.0	64.0	458.4	514.8	49.3	36.1	81.6	43.6	785.8	805.1	214.2	194.9
	b_2	181.8	200.5	504.7	468.4	5.5	36.1	401.4	379.7	20.7	19.4	73.2	96.3	819.9	791.8	180.1	208.2
	b_3	157.8	159.5	337.9	270.6	162.1	87.3	391.9	498.9	13.9	21.7	94.2	121.4	824.3	715.3	175.7	284.7
c_3	b_1	214.1	213.6	348.0	253.8	27.4	87.8	508.8	530.7	64.9	54.2	50.9	73.7	795.4	762.6	204.6	237.4
	b_2	181.6	193.3	386.3	574.5	166.4	18.4	363.6	338.7	19.2	16.7	64.5	51.7	805.5	847.9	194.5	152.1
	b_3	173.9	191.8	466.2	401.9	36.1	0.0	391.0	516.3	13.0	33.4	93.7	48.5	794.2	811.4	205.8	188.6
c_4	b_1	221.0	238.6	325.3	336.7	0.0	29.3	591.1	515.8	17.2	38.8	66.4	79.4	815.1	774.6	184.9	225.4
	b_2	214.2	215.8	416.9	494.0	57.6	22.1	400.4	350.8	64.0	46.5	61.2	86.7	803.5	800.6	196.5	199.4
	b_3	2 05.9	204.2	403.4	564.5	62.0	0.0	350.8	342.8	111.1	16.0	72.7	76.7	785.9	825.8	214.1	174.2
$\bar{X}\ A_1$		188.7^b		395.0^b		51.3^a		439.9^b		37.8^a		76.5^b		795.6^a		204.4^b	
$\bar{X}\ A_2$		197.9^a		406.9^a		37.6^b		445.4^a		28.4^b		81.7^a		782.9^b		217.1^a	
$\bar{X}\ B_1$		212.6^a		338.9^c		26.1^c		528.9^a		39.7^a		66.6^c		788.7^b		211.3^b	
$\bar{X}\ B_2$		190.7^b		462.0^a		51.0^b		378.9^c		29.4^b		79.4^b		803.0^a		196.5^c	
$\bar{X}\ B_3$		176.6^c		401.9^b		56.2^a		420.0^b		30.3^b		91.3^a		782.9^c		217.1^a	
$\bar{X}\ C_1$		175.7^d		386.2^c		34.1^c		463.0^a		23.1^d		93.7^a		772.7^d		227.3^a	
$\bar{X}\ C_2$		186.1^c		389.0^c		59.2^a		440.9^b		26.9^c		85.1^b		790.4^c		209.6^b	
$\bar{X}\ C_3$		194.7^b		405.1^b		56.0^b		441.5^b		33.6^b		63.8^d		802.8^a		197.2^d	
$\bar{X}\ C_4$		216.6^a		423.5^a		28.5^d		425.3^c		48.9^a		73.9^c		800.9^b		199.1^c	

CP- Crude protein; PA- Non protein nitrogen, NPN × 6.25; PB_1- Protein which soluble in phosphate-borate buffer and are rapidly degraded in the rumen; PB_2- Protein which is insoluble in the buffer but is soluble in neutral detergent solution; PB_3 - Insoluble in the buffer and in neutral detergent but is soluble in acid detergent solution; PC- Protein that is insoluble in acid detergent solution; RDP- Rumen degradable protein; RUP- Rumen undegradable protein; a_1- K 28; a_2- G+13R+CZ; b_1- FB - Full bud; b_2- EBL- Early bloom; b_3- MBL- Mid bloom; c_1- I cut ; c_2- II cut; c_3- III cut; c_4- IV cut; Different letters denote significantly different means ($p<0.01$).

Table 5: Correlation analysis between different fractions of proteins in alfalfa cultivars.

	CP	PA	PB_1	PB_2	PB_3	PC	RDP	RUP
CP	1.000							
PA	-0.032	1.000						
PB_1	-0.380	-0.347	1.000					
PB_2	0.276	**-0.747****	-0.287	1.000				
PB_3	0.496	-0.169	-0.016	-0.018	1.000			
PC	**-0.615****	-0.072	0.210	-0.219	-0.352	1.000		
RDP	0.293	**0.592****	-0.121	-0.338	-0.058	**-0.650****	1.000	
RUP	-0.293	**-0.592****	0.121	0.338	0.058	**0.650****	-1.000	1.000

CP- Crude protein; PA- Non protein nitrogen, NPN × 6.25; PB_1- Protein which soluble in phosphate-borate buffer and are rapidly degraded in the rumen; PB_2- Protein which is insoluble in the buffer but is soluble in neutral detergent solution; PB_3- Insoluble in the buffer and in neutral detergent but is soluble in acid detergent solution; PC- Protein that is insoluble in acid detergent solution; RDP- Rumen degradable protein; RUP- Rumen undegradable protein. **- Marked values are stastically significant at p<0.01.

and RDP. On the second axis (27% of the total variation), there was also a contrast PA, PB_1 and PC versus CP, PB_2 and PB_3 (Fig 1). For both cultivars, the PCA indicated a close relationship between the RUP and PC fraction. This is confirmed by the high positive and significant correlations between RUP and PC fraction. Furthermore, RUP value was negatively correlated with PA fraction.

The present study gives a deeper insight on the changes in CP fractions during the growth period of forage legume species which may be used to optimize the management of forage legumes. The decline in protein concentration with advancing maturity occurs both because of decreases in protein in leaves and stems and because stems, with their lower protein concentration, make up a larger portion of the herbage in more mature forage.

However, the earlier reports by Elizalde *et al.* (1999) indicated that the protein fraction PA was not influenced by forage maturity. Further, it was reported that neither the forage species nor maturity of the crop had an impact on fraction PA content. Our results show that proportions of PA fraction in alfalfa was not static, but changes with maturity. After flowering the remobilization of the stored N in the vegetative plant parts take place (Hirel *et al*., 2007) and consequently, the proportion of CP fraction PA decreases.

Soluble protein fractions PA and PB_1 are rapidly degraded in the rumen and available in the RDP pool (Sniffen *et al*., 1992). The higher concentration of fraction PB_1 explains the higher total soluble CP in alfalfa. The results obtained by Yu *et al.* (2003) indicated that alfalfa had a highly rapidly degradable PA fraction and that fraction PB_1 is the lowest, except at the third stage of development, which is in agreement with our results. Insoluble protein fraction PB_2 is presumed to have an intermediate ruminal degradation rate and PB_3 a slow ruminal degradation rate. Varying amounts of these two rumen-insoluble fractions escape ruminal degradation and move to the lower digestive tract (Lanzas *et al*., 2007; Sniffen *et al*., 1992). In our study, values for PB_2 in alfalfa were the largest PB fraction and higher than those reported by Sniffen *et al*. (1992). Undegradable protein fraction PC is regarded as completely unavailable for the ruminanat. PC of investigated alfalfa cultivars in our study were substantially higher than earlier reported values for alfalfa harvested at different growth stages (Yari *et al*., 2012).

Our results confirm that there is significant variability in protein fractions and protein degradability among alfalfa cultivars. Tremblay *et al.* (2000) reported differences among 27 alfalfa cultivars for whole plant *in vitro* RUP but protein fractions were not measured. In the study conducted by Tremblay *et al.* (2003) fractions PB_2, PB_3 and PC accounted for 494, 22 and 41 g kg^{-1} CP, respectively. On the other hand, differences in plant RUP cannot always be atributed to leaf and stem RUP. Hence, plant RUP concentration is not only a function of leaf and stem RUP, but also depends on the proportion of leaves and stems. Results obtained in the investigation of Tremblay *et al.* (2003) showed that RUP concentration was, on average 15% higher in leaves than in stems.

CONCLUSION

In conclusions, the proportion of the CP fractions of alfalfa varies during the growth period with substantial differences between cultivars, stage of maturity and cuts. From a nutritional and breeding point of view, cultivar such as G + 13R + CZ are desirable because it combine high CP values with low protein degradability. Selection of such cultivars should aid in the development of populations with higher protein of better quality for ruminant nutrition. Our results strongly suggest that protein fractions of the CNCPS should be considered as a reliable alternative laboratory method for *in vitro* RUP to screen genotypes for breeding purposes. In general, the chemical CP fractionation valuable information in adition to the classical charcteristics such as energy or fibre content, aids in better evaluation of the quality of forage legume species. Moreover, the present study provides valuable data for the modelling of the CP fractions, which should be the aim of future investigations.

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