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Determination of ruminal protein degradation of three forages using *in vitro* protein fractions and *in situ* protein degradability characteristics

Abstract

The objective of this study was to determine the crude protein fractions of selected three forages (A, B_1, B_2, B_3 and C) by *in vitro* Cornell Net Carbohydrate and Protein System (CNCPS) and the crude protein degradability charecteristics by *in situ* Nylon Bag Technique (NBT). Also, ruminal protein degradabilities were compared according to the feeding levels of ruminant to gain a better understanding of the suitability of these techniques in assessing the forage types. The forages used most commonly in Aegean region were chosen as a feed material: mature alfalfa hay (AH_m), mature grass hay (GH_m), normal maize silage (MS_n) in the study. The soluble protein (SolP), the non-protein nitrogen (NPN, based on SolP%), the neutral detergent insoluble protein (NDIP) and the acid detergent insoluble protein (ADIP) of forages were determined based on CNCPS. Then, the crude protein (CP) fractions, i.e., A=NPN, B_1=fast, B_2=intermediate, B_3=slow and C=not fermented and unavailable to the animal were calculated. *In vitro* degradable intake protein (DIP) was calculated (based on CP% and g/kg DM) by using *in vitro* CP fractions according to dry matter intake fed: DIP_{1X} = at 1x maintenance level of intake, DIP_{2x} =at 2x maintenance level of intake, and DIP_{3x}=at 3x maintenance level of intake. Each forage was incubated between 0-72 h in the rumen of three weathers for three times based on NBT. The CP degradation parameters are (a): fraction of CP immediately soluble protein, (b): the fraction of CP insoluble but degradable in the rumen, (c): the rate constant of degradability of fraction (b). Then, the effective protein degradabilities (based on CP% and g/kg DM) are estimated by using the CP degradation parameters as EPD_2, EPD_5 and EPD_8 assuming rumen outflow rates of 2, 5 and 8 % h^{-1}, respectively. In conclusion, the DIP_{3x} values of MS_n, AH_m and GH_m were 75.06, 63.19 and 56.36 % of CP, respectively. In a different order, the EPD_8 values of AH_m, MS_n and GH_m were found 61.16, 55.88 and 33.75 % CP, respectively. AH_m had the highest ruminal protein degradability (based on g/kg DM) compared to the other two forages both CNCPS and NBT. Both of the methods are much more suitable for AH_m than MS_n and GH_m, because the differences between DIP_{3x} and EPD_8 values were found 3.3, 14.2 and 18.7g/kg DM for AH_m, MS_n and GH_m, respectively.

Keywords: CNCPS parameters, forages, nylon bag technique, protein degradation

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Introduction

Controlling the protein fractions in forages is one way to improve the efficiency of nitrogen use and decrease nitrogen excretion to the environment on ruminant farms.[1] First, it is also known that at least 60% of the ruminant rations come from forages according to the organic livestock standards.[2] Second, protein supplementation to make forages a protein source for the ration formulation represents a large fraction of the cost of ruminant rations. As a result, forage protein analysis comes on the top of the list in accurately formulating rations.[3] Protein evaluation methods such as *in vivo*, *in situ* and *in vitro* are used to determine the ruminal protein degradability or digestibility of forages.[4] Although the conventional *in vivo* method is thought to accurately reflect the feeding value and protein degradability of total rations, it is risky, labour-intensive and expensive.[5] Some alternative methods such as *in situ* Nylon Bag Technique (NBT) and *in vitro* Cornell Net Carbohydrate and Protein System (CNCPS) have become increasingly popular. The NBT is now accepted as one of the basic methods required by the protein evaluation methods proposed by NRC.[6] However, *in situ* values for forages may be affected by microbial contamination of bag residues which significantly reduces the apparent degradability.[7,8] Therefore there is still a need to know the variability of values for rumen degradability values within a given forage type.[9] This fact is a consequence of not accounting for a time lag in passage through the rumen, during which particles may be digested but cannot escape, and this may result in an underestimation of the rumen degradable content.[10] CNCPS, an *in vitro* model, estimates the degradable proteins of the forages using five CP fractions in protein precipitant agents, buffer and detergent solutions (Fox et al. 2003). Briefly, the A fraction is non-protein nitrogen (NPN), the B fraction is degradable true protein and the C fraction is undegradable true protein.[11] Fraction B is divided into three subfractions (B1, B_2 and B_3) based on ruminal degradability rate. However, estimation of protein degradability by this method is still unreliable and requires refinement and standardization. Also, the CP fractionation method requires a much larger data bank before robust regression equations can be formulated for rumen protein degradability estimation.[8]

The methods and mathematical models for ruminants recognize that the ruminal protein degradability of forages may differ by various

factors[1] such as; protein structure, solubility, microbial proteolytic activity, microbial access to the protein, ruminal retention time of dietary protein, stage of maturity and conservation type.[12] The factor is due to the fact that the structure of protein may be altered by the type of conservation (hay vs. silage) and methods used within conservation type, such as pre-ensiling wilting. Thus a more clear understanding of the effect of forage conservation on protein quality will aid in improving the efficiency of N utilization by ruminants and decrease dependency on expensive, protein rich supplements.[8] Unfortunately, not enough studies have been conducted to determine the CP degradability of forages in Turkey based on the alternative methods of *in vivo*.

The purpose of this study is to determine the crude protein fractions of selected three forages (A, B_1, B_2, B_3 and C) by *in vitro* Cornell Net Carbohydrate and Protein System (CNCPS) methods and the crude protein degradability characteristics by *in situ* Nylon Bag Technique (NBT). Also, the *in vitro* degradable intake protein and *in situ* effective protein degradability are compared according to the feeding levels of ruminant to gain a better understanding of the suitability of these techniques in assessing these forages.

Materials and methods

Experimental forages

Three different forage samples which are most commonly used: alfalfa hay (AH), grass hay (GH), maize silage (MS) with tree replicates were collected from Aegean Region of Turkey forages farms. The hays are classified based on their neutral detergent fiber (NDF) contents while MS is classified based on its dry matter (DM) content according to the NRC[6] as follows: mature AH (AH_m, > 46% NDF), mature GH (GH_m, grass x legume mixtures predominantly grass, > 57 % NDF) and normal MS (MS_n, 32-38 % DM)

Methods and mathematical models

The chemical compositions: dry matter (DM), crude ash (CA), crude protein (CP) and ether extract (EE) were determined by Weende analysis method.[13] Ankom Fiber Analyzer (Ankom 200, Ankom Technology, Fairport NY) was used to determine NDF and acid detergent fiber (ADF) analysis.[14] NDF analyses were carried out as alpha amylased pretreated on MS. All chemical analyses of experimental forages were done at least in duplicate. The Van Soest analysis method was used for acid detergent lignin (ADL) analysis.[15] The chemical compositions of experimental forages are shown in Table 1.

Table 1 Chemical composition of experimental forages (based on g/kg DM)

Forages	DM, g/kg	CA	CP	EE	NDF	NFC	ADF	ADL
AH_m	910.7[a]	112.5[a]	160.7[a]	15.1[b]	500.9[b]	210.7[b]	359.1	86.3
GH_m	910.8[a]	123.7[a]	82.7[b]	14.7[b]	612.4[a]	166.5[b]	382.2	69.9
MS_n	350.4[b]	66.8[b]	73.8[b]	23.3[a]	488.7[b]	347.3[a]	297.1	56.5
SE	93.5	10.1	14.7	1.5	23.5	31.5	16.5	6.2
P value	0	0.017	0.002	0.005	0.028	0.016	0.066	0.142

AHm, mature alfalfa hay; GHm, mature grass hay; MSn,normal maize silage ; DM, dry matter; CA, Crude ash; CP, crude protein; EE, Ether extract; NDF, Neutral detergent fiber (maize silage amylase pretreated); NFC, soluble carbohydrates in neutral detergent solution (100 – CA - CP – EE – NDF); ADF:, acid detergent fiber, ADL, acid detergent lignin; SE, Standard error of mean

Different letters (a b, c) in the same row are statistically different.

The *in vitro* CNCPS parameters

The method standardized for the CNCPS parameters of forages, total soluble protein (SolP), NPN (SolP %), neutral detergent insoluble protein (NDIP) and acid detergent insoluble protein (ADIP) were done based on Licitra et al.[16] NDIP and ADIP were determined by filtering NDF and ADF residue on filter paper followed by Kjehdahl method. Then, the CP fractions are calculated as non-protein nitrogen (NPN, A Fraction) and as true proteins (B and C fractions). Fraction A (NPN) is soluble in buffer and tungstic acid. Fraction B is divided into three subfractions (B_1, B_2 and B_3) based on ruminal degradability rate. B_1 (fast) is soluble in buffer and precipitated by tungstic acid. A+ B1 fractions of forages generate the parameter of total soluble proteins (SolP). Fraction B_2 (intermediate) is insoluble in buffer solution but soluble in neutral detergent, fraction B_3 (slow) is soluble in acid detergent but insoluble in neutral detergent, fraction C (not fermented and unavailable to the animal) is insoluble in acid detergent.[11] The following equations were used to calculate the CP fractions of forages: A (% CP) = SolP (% CP) x NPN (SolP%) ; B_1 (% CP) = (SolP (% CP) - A (% CP)) ; C (% CP) = ADIP (% CP) ; B_3 (% CP) = (NDIP(% CP) - ADIP(% CP)) ; B_2 (% CP) = (100 - Fractions (A+ B_1+ B_3+C)) (% CP)

Degradable intake protein (DIP) was calculated by using the following equations: RDPA : rumen soluble protein, A fraction (NPN); RDP B_1 : (B_1 x (Kd_{1x} / Kd_{1x} +K_p B_1)) B_1 fraction (fast soluble protein) : RDPB_2 : (B_2 x (Kd_{1x} / Kd_{1x} +$K_p$$B_2$)) B_2 fraction (intermediate degradable protein) : RDP B_3 : (B_3 x (Kd_{1x} / Kd_{1x} +K_p B_3)) B_3 fraction (slow degradable protein) : RDPTOTAL = RDPA +RDP B_1 + RDPB_2 + RDP B_3 : RDPTOTAL = DIP_{1X} (Degradable intake protein) according to dry matter intake fed at 1x maintenance level). In these calculations (DIP_{1X} = at 1x maintenance level of intake, DIP_{2x} =at 2x maintenance level of intake, and DIP_{3x}=at 3x maintenance level of intake), the values stated in Sniffen et al.,[11] and Fox et al.,[17] were used for the coefficients of outflow rate on the different levels of dry matter intake (K_p) and degradation rate of B fractions (Kd), respectively.

In situ Nylon Bag Technique

The *in situ* nylon bag method procedures were approved by the internal ethical committee of the Ege University (Approval no: 2002/06). Three mature Tahirova wethers (from local Kıvırcık ewes

and imported East Friesian rams, contributing 25% and 75% of the genetic makeup, respectively) were fitted with a rumen cannula (40mm diameter) were used. The wethers fed twice daily at 9.00 pm and 16.00 pm with the diets 60% alfalfa hay and 40% concentrate feed with "*maintenance level x 1.25*". The alfalfa hay contained 145.0gkg^{-1} of CP and 8.00MJkg^{-1} of metabolisable energy (ME), the concentrate contained 150.0g kg^{-1} of CP and 11.50MJkg^{-1} of ME. Vitamin-mineral composition of concentrate consists of following: Vitamin A 7000U/kg, Vitamin D_3 700U/kg, Vitamin E 25mg/kg, Ca 1.1%, P 0.4% and Na 0.25%. The vaccination and parasite applications were done based on veterinary recommendations. The animals were kept individually and had free access to the water. *In situ* CP degradability of forages was determined according to the method of Bhargava and Orskov[18] by using Neway package program. The nylon bags were 9x14 cm in size with pore diameter of 40µm. The forages were grinded using 2.5 mm sieve, weighed 3 g, and then incubated in the Rumen for periods 4, 8, 16, 24, 48 and 72 h. After removal from the rumen, the bags were rinsed in cold tap water. The washing losses were determined by measuring one hour incubation in 39°C water. Then, all bags were washed for 10 min in a washing machine, dried at 55-60°C for 48 h and weighed. Finally, the residues in the bags were used to determine CP degradability. Each feedstuff was tested using three animals with the three replicates (three bags per wether). *In situ* CP degradability was evaluated by "a+b (1- $e^{-c\ t}$)" model.[19] The CP degradation characteristics are a: fraction of CP immediately soluble protein, b: the fraction of CP insoluble but degradable in the rumen, c: the rate constant of degradability of fraction b and t: the time of incubation on the model. Residual standard deviation (RSD) of equation was obtained. Effective protein degradability (EPD values) was calculated using the following equation "a+(bxc/c+k)",[19] where k is the estimated rate of outflow from the rumen to the abomasum. The EPD values are estimated as EPD_2, EPD_5 and EPD_8 assuming rumen outflow rates of 2, 5 and 8 % h^{-1}, which is representative for low, medium and high feeding levels, respectively.

Statistical analysis

The general linear model procedure of statistical package SPSS was used one-way ANOVA on results (SPSS$^{15.0}$ 2005).[20] The Duncan test was used to compare the means, when significant differences observed.

Results

The crude protein fractionation and degradable intake protein values

The *in vitro* CNCPS parameters were shown in Table 2 (based on CP %) and Figure 1 (based on g/kg DM). The AH_m had the highest B_2 and the lowest A (NPN) fraction, and MS_n had the highest SolP, A (NPN) and all DIP values compared to the other two forages ($p<0.05$, Table 2). When CNCPS parameters were calculated based on g/kg DM, AH_m reached the highest values of the parameters because of high CP content of AH_m. GH_m had the lowest A, B_1, B_2 and all DIP values compared to the other two forages (Figure 1).

In situ effective protein degradability characteristics

The CP degradability of forages with the incubation time was ranged between 25.06-83.42 % for 0-72 h (Figure 2). *In situ* CP degradation characteristics are shown in Table 3 (based on CP %) and Figure 3 (based on g/kg DM). MS_n had the highest (a) parameter while the (a) parameter of AH_m was similar to the MS_n. AH_m had the highest (c) parameter compared to other two forages ($p<0.05$). All EPD values had the same pattern and they were different each other being AH_m had the highest values, while GH_m had the lowest values ($p<0.05$).

Table 2 *In vitro* CNCPS parameters of experimental forages (based on % CP)

Forages	CNCPS parameters of crude protein fractions			Crude protein fractions					Degradable intake protein		
	SolP	NPN (SolP, %)	NDIP	A (NPN)	B_1	B_2	B_3	C (ADIP)	DIP_{1x}	DIP_{2x}	DIP_{3x}
AH_m	37.11^b	87.78	31.59^{ab}	32.56c	4.55	31.30^a	17.08^{ab}	14.51	66.65^b	65.01^b	63.19^b
GH_m	42.90^b	94.5	39.30^a	40.60^b	2.3	17.80^b	21.84^a	17.46	58.87^b	57.46^b	56.36^b
MS_n	56.00^a	93.22	22.15^b	52.16^a	3.85	21.84^b	11.58^b	10.59	76.53^a	75.74^a	75.06^a
SE	3.03	1.34	2.86	3.06	0.54	2.36	1.8	1.28	2.8	2.89	2.95
P value	0.003	0.07	0.015	0.003	0.237	0.022	0.032	0.061	0.004	0.004	0.003

AHm, mature alfalfa hay; GHm, mature grass hay; MSn,normal maize silage; SolP, Soluble protein; NPN, nonprotein nitrogen (based on % SolP); NDIP, Neutral detergent insoluble protein;A fraction (NPN), nonprotein nitrogen; B_1, fast soluble protein; B_2, intermediate degradable protein; B_3, slow degradable protein;ADIP (C), acid detergent insoluble protein not fermented and unavailable protein;

DIP, Degradable intake protein fed at 1x maintenance level, at 2x maintenance level of intake, and at 3x maintenance level of intake.

Different letters (a,b,c) in the same row are statistically different

SE, Standard error of mean

Table 3 *In situ* crude protein degradation characteristics of experimental forages (based on % CP)

Forages	Degradation parameters				Effective protein degradability		
	a	b	c, h^{-1}	RSD	EPD_2	EPD_5	EPD_8
AH_m	37.26[a]	46.02	0.0871[a]	1.65	74.41[a]	66.29[a]	61.16[a]
GH_m	21.78[b]	37.99	0.0420[b]	1.66	45.58c	37.62c	33.75c
MS_n	40.34[a]	36.85	0.0617[b]	1.28	67.61[b]	60.11[b]	55.88[b]
SE	2.44	2.18	0.005	0.08	2.45	2.51	2.48
P value	0.001	0.174	0.002	0.09	0	0	0

AHm, mature alfalfa hay; GHm, mature grass hay; MSn,normal maize silage; RSD, Residual standard deviation of equation; SE, Standard error of mean

Degradation parameters : a an intercept representing the proportion of CP solubilized at initiation of incubation time (soluble fraction), b the fraction of CP insoluble but degradable in the rumen, c the rate constant of degradability of fraction b

effective protein degradability (EPD) = a+(bxc/c+k) calculated at rumen outflow rate k = 0.02, 0.05, and 0.08 h^{-1}

Different letters (a,b,c) in the same row are statistically different.

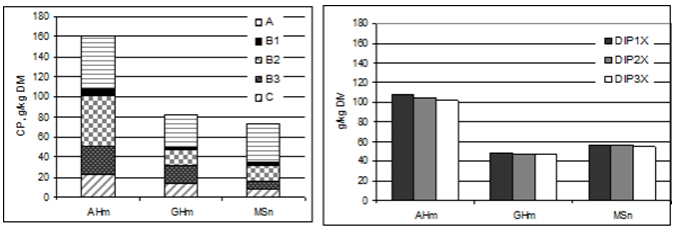

Figure 1 The CNCPS parameters of experimental forages (based on g/kg DM).

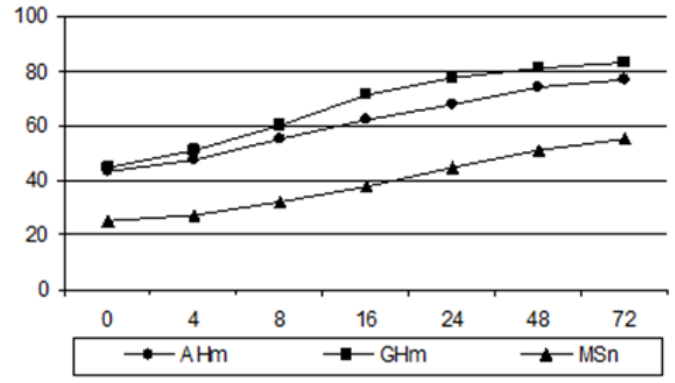

Figure 2 *In situ* crude protein degradability of experimental forages with the incubation time (based on % CP).

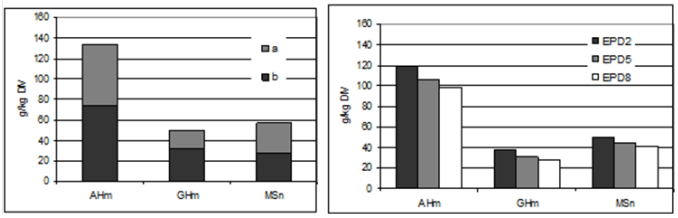

Figure 3 *In situ* crude protein degradability characteristics of experimental forages (based on g/kg DM).

Discussion

AH_m had the highest CP content of forages compared to the other two forages (Table 1). The CP contents of GH_m and MS_n were similar to each other and lower than AH_m ($p<0.05$). The CP contents of forages in our data were slightly lower in MS_n (88.0g/kg DM) and in AH_m (178.0g/kg DM) and lower in GH_m (133.0g/kg DM) than those reported by NRC (2001).[6] According to the statistical analyses, the following trend was apparent. GH_m had the highest NDF content and MS_n had the highest NFC content of forages, as expected. Also, MS_n had the highest EE and the lowest DM values compared to other two forages. The variation in the chemical compositon of all forages could be attributed to the stage of maturity at harvesting, soil type, the varieties and types of forages, preservation method and weather conditions. The chemical composition of present study forages were close to the NRC[6] that mature AH, mature GH and normal MS. As a result of this, the all parameters were compared and discussed with this type of forages on the study.

The CNCPS parameters

The CNCPS parameters were affected by the forage types ($p<0.05$) except NPN (Solp, %), B_1 and C (ADIP) fractions (Table 2). These differences could be attributed to the different protein structure, stage of maturity and preservation methods of forages. The high proportions of SolP, A (NPN) in MS_n as a result of intensive protein hydrolysis during ensiling.[21] Similar to our study, Sniffen et al.,[11] showed that B_1 fraction of forages is very low. Generally, when forages are conserved through ensiling or drying, there is a shift in the proportion of B_1 and B_2 towards A (NPN) in silage and B_3 in dried forages.[8] CNCPS parameters of forages were compared with the values of Fox et al.[17] (CNCPS ver. 5 feedbank) and those determined by Fortina et al.[22] The results of our analysis were generally in agreement with Fox et al. (2003). However, some differences were observed for SolP, A and B_1 fractions of AH and GH, for C fractions of GH. The hays are categorized based on their vegetative stage according to the CNCPS feedback. However in our study, similar to Fortina et al.,[22] this approach was not used, because it was not applicable on the farms where we collected the forage samples. The SolP (CP %) values of AH and GH were changed between 15-30% and 25-26% in CNCPS

feedbank, respectively which is lower than our data 37.11% in AH_m and 42.9% in GH_m. However, the SolP of our data were closer to Fortina et al.[22] results in AH as a 32.5-33.3 %. Also, B_1 fractions of AH and GH were found higher than available data of CNCPS feedbank and Fortina et al.[22] The CNCPS feedbank, MS are subdivided into 5 categories based on the percentage of grain (25%, 35%, 40%, 45% and 50%), whereas in this study we did not consider the different types of MS. The average CP fractions of MS_n in our study resulted similar to the CNCPS feedbank with 50% grain MS. Although the CP values of MS_n were very close to those in Fortina et al.[22] (73.9 versus 89 g/kg DM), B_1 fractions of MS_n in our data were higher than Fortina et al. (2003) (52.16 %CP versus 27.5 %CP). High variations in SolP fractions (thereby B_1 and A fractions) of forages could be due to the maturity and preservation methods. In addition, some authors reported that NPN analyses showed high variability both within and between laboratories due to use of different reagents (tungstic acid vs trichloroacetic acid) and filtration methods.[22,23] The variability of NDIP and ADIP (C) values were caused the difference in B_2 and B_3 fractions of forages. Also, B_2 fraction contains the accumulated analytical error.[23] The C fraction in AH_m and MS_n were close to the values in reported the CNCPS feedbank between 10-25% in AH according to the vegatative stage and 4.5-11.7% in MS, whereas, for GH in the CNCPS feedbank data was lower than our results, respectively between 5.7-8.9%. However, values for GH reported in Fortina et al.,[22] were very close to our findings (17.0% versus 17.9%). Some authors explained that wide variations for the C fractions could be due to the conventional or filter bag methods.[23,24] Also, incorrect technology of silaging occurred leading to heating of ensiled mass and thermal damage of proteins. This caused increase in the C fractions.[21] However; our result of C fraction in MS_n was close to the CNCPS feedbank. DIP values decreased in accordance with the increasing feeding at 1x, 2x and 3x levels of dry matter intake. Similar to our results, Fox et al.,[17] reported that in forages, DIP_{1X} (CP%) was highest at MS_n and lowest at GH_m.

In situ CP characteristics

The (a), (c) parameters and all EPD values were significantly affected by the forages ($p<0.05$) with the exceptions of the (b) parameter. The reported values of the parameter (a) were between 24-50 % for AH,[25,26] between 21-38% for GH[25,27] and 47% for MS.[25] These reported values similar to our results, in that AH_m had the highest, while GH_m had the lowest parameter (a) and all EPD values. The parameter (b) values, reported to be between 32-68% for AH,[25.26] between 26-64% for GH,[25,27] 31 % for MS[25] were close to our result. Comparison of our study with Susmel et al.[25] revealed that the values of the parameter (c) were close in AH (0.0810 h^{-1} versus 0.0871 h^{-1}) and in MS (0.0560 h^{-1} versus 0.0617 h^{-1}). AH_m had the highest (c) parameter in our results. This finding was reported in Karslı et al.,[26] that the (c) parameter in AH (0.1301 h^{-1}) was significantly higher than other forages ($p<0.05$). As the outflow rate (k) increased from the rumen to abomasum (from EPD_2 to EPD_8), the EPD values increased (Table 3). Similar to the Polat et al.,[28] all EPD values were significantly affected by forage type and AH_m had the highest values while GH_m had the lowest values ($p<0.05$).

CNCPS parameters versus *in situ* NBT protein degradability

The DIP_{3x} values (based on CP%) are lined up from highest to lowest MS_n, AH_m and GH_m, whereas EPD_8 values in a different order as AH_m, MS_n and GH_m. This situation, in accordance with the Bach et al.,[29] report on the possibility of lining up the forages in a different order depending on the mathematical models used in determining their CP degradabilities. This fact was explained by Bach et al.,[29] that some of the methods and mathematical models may not be appropriate for all type of forages. On the other hand, when EPD_8 and DIP_{3x} were calculated based on g/kg DM, the forages are lined up same order as AH_m, MS_n and GH_m, because of high CP content of AH_m compared to the other two forages. The differences between DIP_{3x} and EPD_8 (g/kg DM) values were found 3.3, 14.2 and 18.7g/kg DM for AH_m, MS_n and GH_m, respectively. This showed that some forages, like AH_m in our study, are more suitable than others forages for[7,30] to determine ruminal protein degradability.

Conclusion

The ruminal protein degradabilities (based on crude protein percantage) are lined up in as normal maize silage, mature alfalfa hay and mature grass hay by the CNCPS, are lined up in a different order as mature alfalfa hay, normal maize silage and mature grass hay by the *in situ* NBT. Mature alfalfa hay had the highest ruminal protein degradability (based on g/kg dry matter) compared to the other two forages both CNCPS and NBT. Both methods are more suitable for mature alfalfa hay than normal maize silage and mature grass hay. This showed that high protein content could be advantage to determine protein degradability, even different methods are used. Further studies related to analysis of resuduals and fitted and lack-of-fit tests should be performed to asses the accuracy of the models to decribe the protein degradability of forages in Turkey.

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Conflicts of interest

Author declares there is no conflict of interest.

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