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, B, and C) by in vitro Cornell Net Carbohydrate and Protein System (CNCPS) and the crude protein degradability characteristics 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), mature grass hay (GH), normal maize silage (MS) 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=fast, B=intermediate, B=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 = at 1x maintenance level of intake, DIP = at 2x maintenance level of intake, and DIP = 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, EPD, and EPD assuming rumen outflow rates of 2, 5 and 8 % h, respectively. In conclusion, the DIP values of MS, AH, and GH were 75.06, 63.19 and 56.36 % of CP, respectively. In a different order, the EPD values of AH, MS, and GH were found 61.16, 55.88 and 33.75 % CP, respectively. AH 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 than MS and GH, because the differences between DIP, and EPD values were found 3.3, 14.2 and 18.7g/kg DM for AH, MS, and GH, respectively.

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

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. First, it is also known that at least 60% of the ruminal rations come from forages according to the organic livestock standards. Second, protein supplementation to make forages a protein source for the ration formulation represents the organic livestock standards.

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factors 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. 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. 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, B₁, B₂, B₃ 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.

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

<table>
<thead>
<tr>
<th>Forages</th>
<th>DM, g/kg</th>
<th>CA</th>
<th>CP</th>
<th>EE</th>
<th>NDF</th>
<th>NFC</th>
<th>ADF</th>
<th>ADL</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHₐ</td>
<td>910.7a</td>
<td>112.5a</td>
<td>160.7a</td>
<td>15.1a</td>
<td>500.9b</td>
<td>210.7b</td>
<td>359.1</td>
<td>86.3</td>
</tr>
<tr>
<td>GHₐ</td>
<td>910.8a</td>
<td>123.7a</td>
<td>82.7a</td>
<td>14.7a</td>
<td>612.4b</td>
<td>166.5b</td>
<td>382.2</td>
<td>69.9</td>
</tr>
<tr>
<td>MSₐ</td>
<td>350.4b</td>
<td>66.8b</td>
<td>73.8b</td>
<td>23.3b</td>
<td>488.7b</td>
<td>347.3b</td>
<td>297.1</td>
<td>56.5</td>
</tr>
<tr>
<td>SE</td>
<td>93.5</td>
<td>10.1</td>
<td>14.7</td>
<td>1.5</td>
<td>23.5</td>
<td>31.5</td>
<td>16.5</td>
<td>6.2</td>
</tr>
<tr>
<td>P value</td>
<td>0</td>
<td>0.017</td>
<td>0.002</td>
<td>0.005</td>
<td>0.028</td>
<td>0.016</td>
<td>0.066</td>
<td>0.142</td>
</tr>
</tbody>
</table>

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 (SoIP), NPN (SoIP %), neutral detergent insoluble protein (NDIP) and acid detergent insoluble protein (ADIP) were done based on Licitra et al. 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 (B₁, B₂, and B₃) based on ruminal degradability rate. B₁ (fast) is soluble in buffer and precipitated by tungstic acid. A+ B₁ fractions of forages generate the parameter of total soluble proteins (SoLP). Fraction B₂ (intermediate) is insoluble in buffer solution but soluble in neutral detergent, fraction B₃ (slow) is soluble in acid detergent but insoluble in neutral detergent, fraction C (not fermentable and unavailable to the animal) is insoluble in acid detergent. The following equations were used to calculate the CP fractions of forages: A(% CP) = SoIP(% CP) x NPN(SoIP%); B₁ (% CP) = (SoIP(% CP) - A(% CP)); C (% CP) = ADIP(% CP); B₂ (% CP) = (NDIP(% CP) - ADIP(% CP)); B₃ (% CP) = (100 - Fractions (A+B₁+B₂+C)) (% CP)

Degradable intake protein (DIP) was calculated by using the following equations: RDPA : rumen soluble protein, A fraction (NPN); RDPB₁ : (B₁ x (Kd₁ / K + K₁ B₁)) B₁ fraction (fast soluble protein); RDPB₂ : (B₂ x (Kd₂ / K + K₂ B₂)) B₂ fraction (intermediate degradable protein); RDPB₃ : (B₃ x (Kd₃ / K + K₃ B₃)) B₃ fraction (slow degradable protein); RDPTOTAL = RDP A + RDP B₁ + RDPB₂ + RDPB₃; DIP = RDPTOTAL x Kd₁ / K + K₁ B₁ x (Kd₂ / K + K₂ B₂) x (Kd₃ / K + K₃ B₃)) B₃ fraction (degradable intake protein) according to dry matter intake fed at 1x maintenance level. In these calculations (DIP₁ = at 1x maintenance level of intake, DIP₂ = at 2x maintenance level of intake, and DIP₃ = at 3x maintenance level of intake), the values stated in Sniffen et al.,11 and Fox et al.,13 were used for the coefficients of outflow rate on the different levels of dry matter intake (Kd) 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 Kivrck ewes...
and imported East Friesian rams, contributing 25% and 75% of the genetic makeup, respectively) were fitted with a rumen cannula (40 mm 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 145.0 kg\(^{-1}\) of CP and 8.00 MJ kg\(^{-1}\) of metabolisable energy (ME), the concentrate contained 150.0 kg\(^{-1}\) of CP and 11.50 MJ kg\(^{-1}\) of ME. Vitamin-mineral composition of concentrate consists of following: Vitamin A 7000 U/ kg, Vitamin D 700 U/kg, Vitamin E 25 mg/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 9 x 14 cm in size with pore diameter of 40 μm. 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\(^{-t}\))" model.\(^{18}\) 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: 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)\(^{-1}\)\(^{15}\), where k is the estimated rate of outflow from the rumen to the abomasum. The EPD values are estimated as EPD\(_a\), EPD\(_b\) and EPD\(_c\) 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\(^{15,20}\) 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\(_n\) had the highest B\(_n\) 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\(_n\) reached the highest values of the parameters because of high CP content of AH\(_n\). GH\(_n\) had the lowest A, B, B\(_n\) 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\(_n\) was similar to the MS\(_n\), AH\(_n\) 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\(_n\) had the highest values, while GH\(_n\) had the lowest values (p<0.05).

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

<table>
<thead>
<tr>
<th>Forages</th>
<th>CNCPS parameters of crude protein fractions</th>
<th>Crude protein fractions</th>
<th>Degradable intake protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SoIP</td>
<td>NPN (%SoIP, %)</td>
<td>NDIP</td>
</tr>
<tr>
<td>AH(_n)</td>
<td>37.11(^{a})</td>
<td>87.78</td>
<td>31.59(^{a})</td>
</tr>
<tr>
<td>GH(_n)</td>
<td>42.90(^{a})</td>
<td>94.5</td>
<td>39.30(^{a})</td>
</tr>
<tr>
<td>MS(_n)</td>
<td>56.00(^{a})</td>
<td>93.22</td>
<td>22.15(^{a})</td>
</tr>
<tr>
<td>SE</td>
<td>3.03</td>
<td>1.34</td>
<td>2.86</td>
</tr>
<tr>
<td>P value</td>
<td>0.003</td>
<td>0.07</td>
<td>0.015</td>
</tr>
</tbody>
</table>

AH\(_n\), mature alfalfa hay; GH\(_n\), mature grass hay; MS\(_n\), normal maize silage; SoIP, Soluble protein; NPN, nonprotein nitrogen (based on % SoIP); NDIP, Neutral detergent insoluble protein; A fraction (NPN), nonprotein nitrogen; B\(_n\), fast soluble protein; B\(_h\), intermediate degradable protein; B\(_s\), 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

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

<table>
<thead>
<tr>
<th>Forages</th>
<th>Degradation parameters</th>
<th>Effective protein degradability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>AHₘₙ</td>
<td>37.26⁴</td>
<td>46.02</td>
</tr>
<tr>
<td>GHₘₙ</td>
<td>21.78⁴</td>
<td>37.99</td>
</tr>
<tr>
<td>MSₙ</td>
<td>40.34⁴</td>
<td>36.85</td>
</tr>
<tr>
<td>SE</td>
<td>2.44</td>
<td>2.18</td>
</tr>
<tr>
<td>P value</td>
<td>0.001</td>
<td>0.174</td>
</tr>
</tbody>
</table>

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

Degradation parameters: a 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.

Discussion

AHₘₙ had the highest CP content of forages compared to the other two forages (Table 1). The CP contents of GHₘₙ and MSₙ were similar to each other and lower than AHₘₙ (p<0.05). The CP contents of forages in our data were slightly lower in MSₙ (88.0g/kg DM) and in AHₘₙ (178.0g/kg DM) and lower in GHₘₙ (133.0g/kg DM) than those reported by NRC (2001).

According to the statistical analyses, the following trend was apparent. GHₘₙ had the highest NDF content and MSₙ had the highest NFC content of forages, as expected. Also, MSₙ had the highest EE and the lowest DM values compared to other two forages. The variation in the chemical composition 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 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, %), B₁ 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 MSₙ as a result of intensive protein hydrolysis during ensiling. Similar to our study, Sniffen et al., showed that B₁ fraction of forages is very low. Generally, when forages are conserved through ensiling or drying, there is a shift in the proportion of B₁ and B₂ towards A (NPN) in silage and B₂ in dried forages. CNCPS parameters of forages were compared with the values of Fox et al. (CNCPS ver. 5 feedbank) and those determined by Fortina et al. The results of our analysis were generally in agreement with Fox et al. (2003). However, some differences were observed for SolP, A and B₁ 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., 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
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feedback, respectively which is lower than our data 37.11% in AH and 42.9% in GH. However, the SolP of our data were close to Fortina’s 

25
28
25
23,24
3x
m
m
n.

n
3x
25
n
characteristics.

25,26
m
m
n.

n
3x
25

The 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 (CP%) was highest at MS and lowest at GH.

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,25,28 between 21-38% for GH25,27 and 47% for MS.25 These reported values similar to our results, in that AH had the highest, while GH 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 MS25 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 h1 versus 0.0871 h1) and in MS (0.0560 h1 versus 0.0617 h1). AH had the highest (c) parameter in our results. This finding was reported in Karsh et al.,26 that the (c) parameter in AH (0.1301 h1) was significantly higher than other forages (p<0.05). As the outflow rate (k) increased from the rumen to abomasum (from EPD to EPD), the EPD values increased (Table 3). Similar to the Polat et al.,28 all EPD values were significantly affected by forage type and AH had the highest values while GH had the lowest values (p<0.05).

CNPCS parameters versus in situ NBT protein degradability

The DIP values (based on CP%) are lined up from highest to

lowest MS and GH. This situation, in accordance with the Bach et al.,29 report on the possibility of listing 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 and DIP were calculated based on g/kg DM, the forages are lined up same order as AH, MS and GH, because of high CP content of AH compared to the other two forages. The differences between DIP and EPD (g/kg DM) values were found 3.3, 14.2 and 18.7g/kg DM for AH, MS and GH, respectively. This showed that some forages, like AH, in our study, are more suitable than others forages to determine ruminal protein degradability.

Conclusion

The ruminal protein degradabilities (based on crude protein percentage) are lined up in as normal maize silage, mature alfalfa hay and mature grass hay by the CNPCS, 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 CNPCS 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 residuals and fitted and lack-of-fit tests should be performed to asses the accuracy of the models to describe the protein degradability of forages in Turkey.

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Conflicts of interest

Author declares there is no conflict of interest.

References


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