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 ruminant rations come from forages according to the organic livestock standards. 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 the CP fractions of ruminants. Therefore there is still a need to know the variability of values for rumen degradability values within a given forage type. 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. 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. Fraction B is divided into three subfractions (B1, B2, and B3) 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. The methods and mathematical models for ruminants recognize that the ruminal protein degradability of forages may differ by various microbial contamination of bag residues which significantly reduces the apparent degradability. Therefore there is still a need to know the variability of values for rumen degradability values within a given forage type.
Determination of ruminal protein degradation of three forages using in vitro protein fractions and in situ protein degradability characteristics

The purpose of this study is to determine the crude protein fractions of selected three forages (A, B1, B2, 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.

The chemical compositions: dry matter (DM), crude ash (CA), crude protein (CP) and ether extract (EE) were determined by the Van Soest analysis method.15 Ankom Fiber Analyzer (Ankom 200, Ankom Technology, Fairport NY) was used to determine NDF and acid detergent fiber (ADF) analysis.16 NDF analyses were carried out as alpha amylase 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.

### 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 as follows: mature AH (AH > 46% NDF), mature GH (GH > 57 % NDF) and normal MS (MS > 32-38 % DM).

#### Methods and mathematical models

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 (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 fermented and unavailable to the animal) is insoluble in acid detergent.15 The following equations were used to calculate the CP fractions of forages:

\[
\begin{align*}
A & = \text{SolP} \times \text{NPN} \\
B_1 & = \text{SolP} - A \\
B_2 & = B_1 + C \\
C & = \text{ADIP}
\end{align*}
\]

Degradable intake protein (DIP) was calculated by using the following equations: RDP_A : rumen soluble protein, A fraction (NPN); RDP_B1 : (B1 x (Ka_1 / (Ka_1 + K_b_1))) B1 fraction (fast soluble protein); RDP_B2 : (B2 x (Ka_2 / (Ka_2 + K_b_2)) B2 fraction (intermediate degradable protein); RDP_B3 : (B3 x (Ka_3 / (Ka_3 + K_b_3)) B3 fraction (slow degradable protein); RDP_TOTAL = DIP_A + RDP_B1 + RDP_B2 + RDP_B3 ; RDP_TOTAL = DIP_A (Degradable intake protein) according to dry matter intake fed at 1x maintenance level). In these calculations (DIP_A = at 1x maintenance level of intake, DIP_A = at 2x maintenance level of intake, and DIP_A = at 3x maintenance level of intake), the values stated in Sniffen et al.,11 and Fox et al.,12 were used for the coefficients of outflow rate on the different levels of dry matter intake (Ko) and degradadion rate of B fractions (Kd), respectively.

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

| Forages   | DM, g/kg | CA, % CP | CP, % CP | EE, % CP | NDF, % CP | NFC, % CP | ADL, % CP |
|-----------|----------|----------|----------|----------|-----------|-----------|-----------|
| AH_n      | 910.7    | 112.5    | 160.7    | 15.1     | 500.9     | 210.7     | 359.1     |
| GH_n      | 910.8    | 123.7    | 82.7     | 14.7     | 612.4     | 166.5     | 382.2     |
| MS_n      | 350.4    | 66.8     | 73.8     | 23.3     | 488.7     | 347.3     | 297.1     |
| SE        | 93.5     | 10.1     | 14.7     | 1.5      | 23.5      | 31.5      | 16.5      |
| P value    | 0        | 0.017    | 0.002    | 0.005    | 0.028     | 0.016     | 0.066     |
| ADP(%)CP  | 100 x (A+B1+B2+C) (%) |

Different letters (a,b,c) in the same row are statistically different.
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 145.0g/kg of CP and 8.00MJkg⁻¹ of metabolisable energy (ME), the concentrate contained 150.0g/kg of CP and 11.50MJkg⁻¹ of ME. Vitamin-mineral composition of concentrate consists of following; Vitamin A 7000IU/kg, Vitamin D₃ 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. 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⁻ᶜᵗ)" model. The CP degradration 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 degradation 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+x))", where k is the estimated rate of outflow from the rumen to the abomasum. The EPD values are estimated as EPD₂, EPD₃ and EPD₄ 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 16.0 2005). 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 AHₜₙ had the highest B₃ and the lowest A (NPN) fraction, and MSₙ 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, AHₜₙ reached the highest values of the parameters because of high CP content of AHₜₙ. GHₜₙ had the lowest A, B₃, B₃ 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 degradration characteristics are shown in Table 3 (based on CP %) and Figure 3 (based on g/kg DM). MSₙ had the highest (a) parameter while the (a) parameter of AHₜₙ was similar to the MSₙ. AHₜₙ 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 AHₜₙ had the highest values, while GHₜₙ 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) | B₃ | B₃ | C (ADIP) | DIPₓ₁ | DIPₓ₂ | DIPₓ₃ |
|AHₜₙ     | 37.11 | 87.78 | 31.59 | 32.56c | 4.55 | 31.30 | 17.08 | 14.51 | 66.65 | 65.01 | 63.19 |
|GHₜₙ     | 42.90 | 94.5  | 39.30 | 40.60 | 2.3  | 17.80 | 21.84 | 17.46 | 58.87 | 57.46 | 56.36 |
|MSₙ      | 56.00 | 93.22 | 22.15 | 52.16 | 3.85 | 21.84 | 11.58 | 10.59 | 76.53 | 75.74 | 75.06 |
|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 |

AHₜₙ, mature alfalfa hay; GHₜₙ, 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; B₃, fast soluble protein; B₃, intermediate degradable protein; B₃, 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)

| Forages | Degradation parameters | Effective protein degradability |
|---------|-------------------------|---------------------------------|
|         | a | b | c, h<sup>-1</sup> | RSD | EPD<sub>1</sub> | EPD<sub>2</sub> | EPD<sub>3</sub> |
| AH<sub>m</sub> | 37.26<sup>a</sup> | 46.02 | 0.0871<sup>a</sup> | 1.65 | 74.41<sup>a</sup> | 66.29<sup>b</sup> | 61.16<sup>c</sup> |
| GH<sub>m</sub> | 21.78<sup>b</sup> | 37.99 | 0.0420<sup>b</sup> | 1.66 | 45.58c | 37.62c | 33.75c |
| MS<sub>n</sub> | 40.34<sup>a</sup> | 36.85 | 0.0617<sup>b</sup> | 1.28 | 67.61<sup>a</sup> | 60.11<sup>b</sup> | 55.88<sup>c</sup> |
| 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 |

AH<sub>m</sub>, mature alfalfa hay; GH<sub>m</sub>, mature grass hay; MS<sub>n</sub>, 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<sup>-1</sup>

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

Discussion

AH<sub>m</sub> had the highest CP content of forages compared to the other two forages (Table 1). The CP contents of GH<sub>m</sub> and MS<sub>n</sub> were similar to each other and lower than AH<sub>m</sub> (p<0.05). The CP contents of forages in our data were slightly lower in MS<sub>n</sub> (88.0g/kg DM) and in AH<sub>m</sub> (178.0g/kg DM) and lower in GH<sub>m</sub> (133.0g/kg DM) than those reported by NRC (2001). 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 (Solfp, %), B<sub>1</sub> 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 Solfp, A (NPN) in MS<sub>n</sub> as a result of intensive protein hydrolysis during ensiling. Similar to our study, Sniffen et al., showed that B<sub>1</sub> fraction of forages is very low. Generally, when forages are conserved through ensiling or drying, there is a shift in the proportion of B<sub>1</sub> and B<sub>2</sub> towards A (NPN) in silage and B<sub>1</sub> 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 Solfp, A and B<sub>1</sub> 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 Solfp (CP %) values of AH and GH were changed between 15-30% and 25-26% in CNCPS.
feedback, respectively which is lower than our data 37.11% in AH and 42.9% in GH. However, the SolP of our data were closer to Fortina et al.'s results in AH as a 32.5-33.3%. Also, B fractions of AH and GH were found higher than available data from CNCPS feedback and Fortina et al. The CNCPS feedback, 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 in our study resulted similar to the CNCPS feedback with 50% grain MS. Although the CP values of MS were very close to those in Fortina et al. (73.9 versus 89 g/kg DM), B fractions of MS in our data were higher than Fortina et al. (2003) (52.16 %CP versus 27.5 %CP). High variations in SolP fractions (thereby B 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. The variability of NDIP and ADIP (C) values were caused the difference in B and B fractions of forages. Also, B fraction contains the accumulated analytical error. The C fraction in AH and MS at 24-50 % in AH and 4.5-17.7% in MS, whereas, for GH in the CNCPS feedback data was lower than our results respectively between 5.7-8.8%. However, values for GH reported in Fortina et al., were very close to our findings (17.0% versus 17.9%). Some authors explained that this variation for the C fractions could be due to the conventional or filter bag methods. Also, incorrect technology of silaging occurred leading to heating of ensiled mass and thermal damage of proteins. This caused increase in the C fractions. However; our result of C fraction in MS was close to the CNCPS feedback. 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., 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, between 21-38% for GH and 47% for MS. 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, 26-64% for GH, 31 % for MS were close to our result. Comparison of our study with Susmel et al. 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). The highest (c) parameter in our results. This finding was reported in Karsh et al., 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 to EPD), the EPD values increased (Table 3). Similar to the Polat et al., all EPD values were significantly affected by forage type and AH had the highest values while GH had the lowest values (p<0.05).

CNCPS parameters versus in situ NBT protein degradability

The DIPn values (based on CP%) are lined up from highest to lowest MS, AH and GH whereas EPDn values in a different order as AH, MS and GH. This situation, in accordance with the Bach et al., 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., that some of the methods and mathematical models may not be appropriate for all type of forages. On the other hand, when EPD and DIPn were calculated based on kg/kg DM, the forages are lined up in same order as AH, MS and GH, because of high CP content of AH compared to the other two forages. The differences between DIPn 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 for 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 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 kg/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 residuals and fitted and lack-of-fit tests should be performed to assess 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.

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