KNOWLEDGE – International Journal
Vol.34.3
September, 2019

# CONTENT OF TOTAL NITROGEN AND PROTEINS FROM ALFALFA (*Medicago sativa* L.) COLLECTED IN THREE SLOPES

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**Abstract:** Alfalfa (*Medicago sativa* L.) leaves and stems contain different proteins and nitrogen concentration in different stages of growth. The objective of this study is to determine the dynamic of nutrient accumulation of total nitrogen and proteins in leaves and stems. The experiment was conducted in three slopes, on three regions in the Republic of North Macedonia (Tetovo, Skopje and Ovche Pole). Chemical analysis of total nitrogen and proteins were obtained from first, second and third slope. Modern techniques have been used for analyzing the protein activity of plant material in multiple measuring points. Significant differences are found in the production of total nitrogen and proteins between the locations in Tetovo region on one side, and Skopje and Ovche Pole on the other side. It shows that Tetovo region has better conditions for producing alfalfa protein. Alfalfa is a culture that is rich in high nitrogen and protein content in the Tetovo region, which is correlated with the amino acid composition, resulting in a high biological value. Therefore, alfalfa is the dominant forage crop and active diet culture with high applicability to the bio-diet. Proteins are the most abundant biomolecules in plants and other organisms. Protein macromolecules make up half of the dry matter in the plant cell. The plant cell contains many different proteins with a specific function. Proteins contain the most important property - biological specificity, so the individuality of each organism is conditioned by the type of protein it is made of. Proteins have a specific structure that is found in their biological activity. Proteins are the most important components in the plant cell. Nitrogen is one of the many compounds important for plant life processes and its role in physiological processes in plants is quite large. The needs of certain plants for nitrogen are different. Nitrogen in plants is important in the composition of proteins, nucleic acids, coenzymes, alkaloids, some pigments and other compounds. Accordingly, the nitrogen in plants exists as non-protein and protein nitrogen, found in the protein component. It can only enter the plant cell if it is reduced to ammonia. This scientific research paper provides a comprehensive analysis of the nitrogen and protein composition of alfalfa grown in the Skopje, Tetovo and Ovche Pole region. The results of this research, represent the first full and complete overview of alfalfa (*Medicago sativa* L.), with its protein composition, which would be of great importance for the further cultivation of this forage crop. Scientific evidence has shown that the Tetovo region has a higher advantage over the Skopje and Ovche Pole region in terms of nitrogen and protein content, which are crucial nutrients in forage crops.
**Keywords:** Kjeldhal method, Skopje, Tetovo and Ovhce Pole region, forage.

## 1. INTRODUCTION

Alfalfa (*Medicago sativa* L.) is one of the oldest forage plants and, because it is perennial, shows high yields with a high quality. It can regenerate rapidly and provides five to six crops during a vegetation season. According to biomass dynamics and nutritional value, alfalfa is one of the most important forage crops in the world due to its high quality, yield and adaptability to different climatic conditions (Gashaw and Harmoniz 2015). Alfalfa has a high concentration of protein with a favourable amino acid composition, resulting in a high biological value. It also contains high amounts of important vitamins, carbohydrates, saponins and mineral elements, especially calcium. In addition, important chemical elements and other active components, essential for the growth and development of animals, are present in alfalfa (Hao et al., 2008). Alfalfa adds nitrogen to the soil and improves the soil fertility (Arshad et al., 2016). All the most important biochemical and assimilation processes relate to phosphorus availability. In the old alfalfa stands the phosphorus availability becomes lower and, consequently, alfalfa forage yield decreases extensively (Madani et al., 2014). In this sense, alfalfa is a dominant fodder culture and an active dietary culture with high applicability in bio-nutrition. Alfalfa is an important livestock feed grown worldwide. The

United States is the fourth largest producer of alfalfa behind corn, wheat and soybeans of all cultivars (Fernandez-Cornejo et al., 2016). Alfalfa is used as a perennial culture continuously for 4-5 years, providing four to five slopes per year. In temperate regions, slopes should be taken every 4-6 weeks. Alfalfa plays an important role in crop rotation and provides large quantities of organic matter to the soil, thereby improving the physical, chemical and microbiological properties of the soil as well as the soil structure. As a nitrogen-fixing plant it enriches the soil with readily available nitrogen, which makes alfalfa an excellent pre-culture for numerous cereals, industrial and horticultural crops. Unlike most other fodder legumes, alfalfa is usually grown in pure monocultures, although it can be mixed with other legumes and grasses.

The constant chemical composition of alfalfa, which could also be maintained by the application of organic acids, enabled the yield of alfalfa to be increased (Ke et al., 2017).

Alfalfa makes an ideal protein supplement. The proteins in alfalfa are highly digestible and available in the rumen to feed the rumen microbes. Because of this, it stimulates digestion of the fiber in both alfalfa and the low-quality forage. This stimulation of digestion also increases intake of the low-quality forage, and as result improves the total digested nutrients that the domestic animals receives (Koleva Gudeva, 2010).

In Macedonia, this crop is grown on an area of 19,000 ha, with an average yield of 6-6.5 t/ha (Илиевски, 2013).

The role of alfalfa (Medicago sativa L.), as the highest quality forage culture, in the development of agricultural production and the intensification of forage production is due to the ability to ensure high yield, to have the ability to regenerate continuously and to possess high nutritional value (Butleska Gjoroska, et all, 2019).

## 2. PLANT MATERIAL AND METHODS

### 2.1. Plant material

Plant material from alfalfa (*Medicago sativa* L.) was collected from three different regions in the territory of the Republic of North Macedonia: Tetovo region, Skopje region and the Ovche Pole region, from 19 different locations in three slopes. The material was collected during the vegetative cycle (from June to August) in 2013. In the first, second and third slope, plants were collected on June 15, July 17 and August 15, respectively (Table 1). Analysis of nitrogen and proteins was conducted on dry plant material using the neutralization method.

***Table 1. Description of the locations from the examined locations altitude (m) and latitude ($^{O}N$) and longitude ($^{O}E$) with the dates of first, second and third slope***

| No. | Location | Region | Altitude (m) | Latitude (°N) | Longitude (°E) | First slope (date) | Second slope (date) | Third slope (date) |
|---|---|---|---|---|---|---|---|---|
| 1. | Bogovinje | Tetovo | 531.50 | 41.9236809 | 20.9168772 | 15.06.2013 | 16.07.2013 | 17.08.2013 |
| 2. | Vrutok | Tetovo | 682.41 | 41.7665300 | 20.8381550 | 15.06.2013 | 16.07.2013 | 17.08.2013 |
| 3. | Dzepchishte | Tetovo | 474.48 | 42.0331690 | 21.0001650 | 15.06.2013 | 16.07.2013 | 17.08.2013 |
| 4. | Galate | Tetovo | 600.73 | 41.8381370 | 20.8813700 | 15.06.2013 | 16.07.2013 | 17.08.2013 |
| 5. | Zelino | Tetovo | 1605.94 | 41,9006530 | 21.1175770 | 15.06.2013 | 16.07.2013 | 17.08.2013 |
| 6. | Pechkovo | Tetovo | 991.87 | 41.7843700 | 20.8311530 | 15.06.2013 | 16.07.2013 | 17.08.2013 |
| 7. | Jegunovce | Tetovo | 658.34 | 42.1245655 | 21.0875064 | 15.06.2013 | 16.07.2013 | 17.08.2013 |
| 8. | Avtokomanda | Skopje | 246.68 | 42.0006868 | 21.4536642 | 16.06.2013 | 17.07.2013 | 18.08.2013 |
| 9. | Sopishte | Skopje | 1017.16 | 41.8638490 | 21.3083500 | 16.06.2013 | 17.07.2013 | 18.08.2013 |
| 10. | Drachevo | Skopje | 264.41 | 41.9352675 | 21.5098515 | 16.06.2013 | 17.07.2013 | 18.08.2013 |
| 11. | Saraj | Skopje | 424.88 | 42.0017493 | 21.2815977 | 16.06.2013 | 17.07.2013 | 18.08.2013 |
| 12. | Radishani | Skopje | 392.32 | 42.0732769 | 21.4479917 | 16.06.2013 | 17.07.2013 | 18.08.2013 |
| 13. | Vlae | Skopje | 256.07 | 42.0072938 | 21.3801924 | 16.06.2013 | 17.07.2013 | 18.08.2013 |
| 14. | Glumovo | Skopje | 274.74 | 41.9817742 | 21.3103747 | 16.06.2013 | 17.07.2013 | 18.08.2013 |
| 15. | Chesinovo | Ovche Pole | 294.00 | 41.8735350 | 22.2905610 | 17.06.2013 | 18.07.2013 | 19.08.2013 |
| 16. | Karbinci | Ovche Pole | 342.98 | 41.7882100 | 22.2622460 | 17.06.2013 | 18.07.2013 | 19.08.2013 |
| 17. | Obleshevo | Ovche Pole | 297.63 | 41.8639320 | 22.2622460 | 17.06.2013 | 18.07.2013 | 19.08.2013 |
| 18. | Lozovo | Ovche Pole | 277.86 | 41.7806752 | 21.8995629 | 17.06.2013 | 18.07.2013 | 19.08.2013 |
| 19. | Mustafino | Ovche Pole | 289.18 | 41.8407190 | 22.0789350 | 17.06.2013 | 18.07.2013 | 19.08.2013 |

### 2.2. Laboratory method

**Determination of the total nitrogen content**

The Kjeldhal method for nitrogen determination is performed in three steps:

*1. Combustion with a catalyst mixture:* In a dry, clean combustion cell, was placeed 1 g of mashed dry plant material and 5 g of catalyst mixture, stirred and added 20ml of concentrated $H_2SO_4$. The incineration was carried out for 2 hours at 410°C and the procedure was completed by discoloration of the contents in the cell.

*2. Distillation of ammonia and its condensation into boric acid:* After combustion, the contents of the test tube were quantitatively transferred to a Kjeldhal flask (500 ml) by rinsing with 70 ml of distilled water. After that was added 70 ml of 40% NaOH solution to the Kjeldhal flask and the distillation with water vapor started. The steam produced in the flask with the heater passes through the tubes and enters the Kjeldhal flask, boiling the solution, and the separated ammonia is carried to the receiver Erlenmeyer flask (250 ml) in which acid is immersed and 2-3 drops of indicator mixture. Distillation takes about 15-20 minutes, until about 150 ml is collected in the Erlenmeyer flask.
*3. Titration with hydrochloric acid:* The distillate was cooled and titrated with 0.1 N HCl solution was conducted, when changing the distillate color from blue to discoloration.
The calculation is as follows: from the amount of bound HCl the total nitrogen content is calculated. 10 ml of 0.1N HCl binds 0.00142 g of nitrogen the calculation was made by the following formula:

$$\text{Tottal N \%} = \frac{\text{a x FHCl x 0.00142 x 100}}{\text{b x (100 − W)}}$$

where:
a - spent ml of 0.1N HCl;
FHCL - solution factor of HCl;
b - measured quantity of dry plant material (g);
0.00142 g of nitrogen correspond to 1 ml of 0.1 N HCl;
W - the sum of the percentage of water content in the sample.

**Determination of *proteins***

The method of determination of proteins is according to the Mohr method and is performed in 4 steps.
1. *Protein separation from other nitrogen compounds*: The first step in determining the protein content of alfalfa using the Mohr method is to separate the protein from the other nitrogenous compounds. This was done by precipitation with a solution of 0.5% acetic acid solution, with aim to remove polypeptides, amides, alkaloids, nitrates and other amino compounds.
2. 3. and 4 the step. The next three steps are the same as the method for determining total nitrogen.
The calculation is as follows: From the amount of bound HCl the protein nitrogen content is calculated. 10 ml of 0.1N HCl binds 0.00142 g of nitrogen. The calculation is carried out using the same formula that calculates the percentage of nitrogen. Protein in alfalfa, as in many other plant crops, has an average of 16% nitrogen, so multiplying the value of protein nitrogen by a factor of 6.25%, the amount of protein is obtained.

$$\text{Protein \%} = \text{total protein N \%} \times 6.25$$

**2.3. Statistical data processing**

The data were analysed (XLSTAT 2014) via one-way variance analysis (ANOVA) to determine the significant differences ($p<0.05$ and $p<0.01$) between the mean values of the samples. Subsequently, the results were post-hoc analysed using Duncan's multiple ranking test to determine statistically significant differences in the contents of total nitrogen and proteins among the three slopes.

**3. RESULATS AND DISCUSION**

**3.1. Total nitrogen**

The average values for total nitrogen at the examined locations, on the level of the regions, in all slopes, at the Republic of North Macedonia, expressed as a percentage is shown in figure 1.
In all slopes separately and in all slopes together, the highest content of total nitrogen in the examined locations was measured in the Tetovo region, at the Dzepchishte location. The smallest content in the first and in the second slope was in Ovche Pole, Mustafino location and in the third slope and in all slopes together, the lowest content was measured at Skopje region, in Drachevo location.

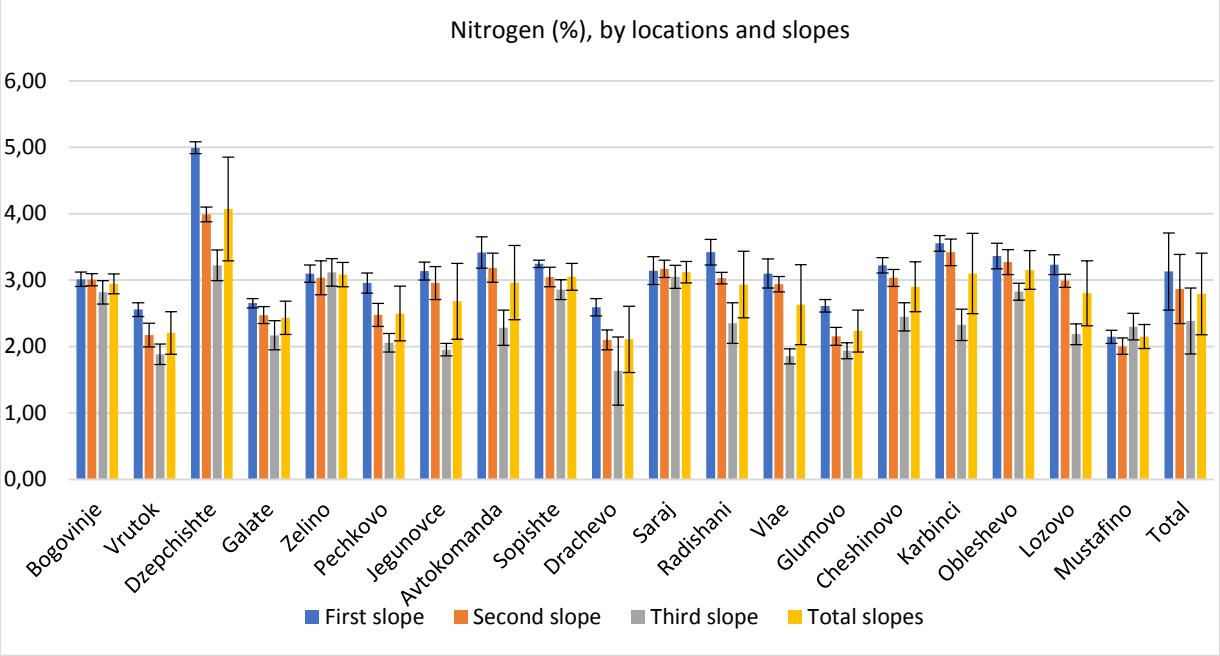


***Figure 1. The content of total nitrogen at the examined locations from the three regions, in the three slopes, expressed in percentage (%) of dry plant material***

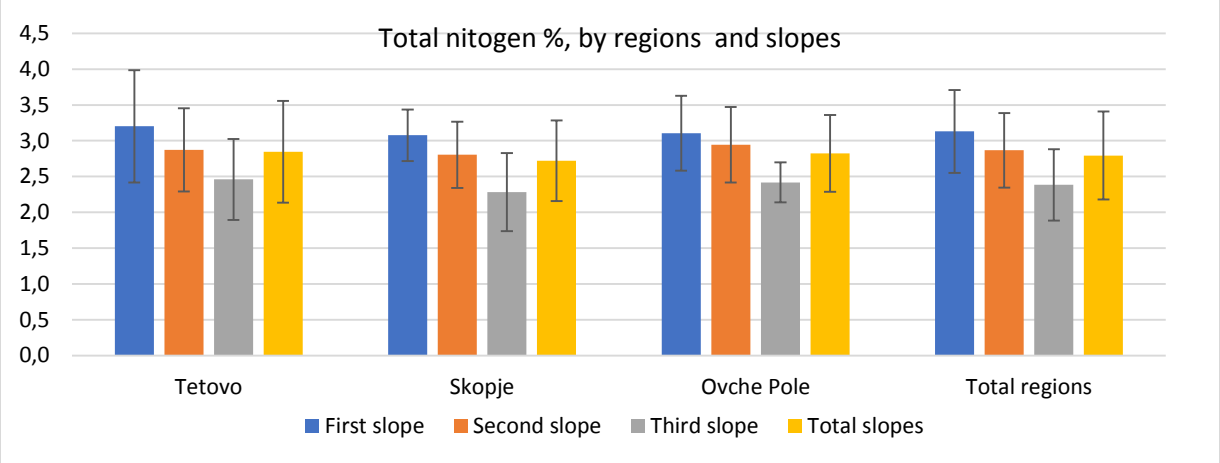


***Figure 2. Total nitrogen content at the examined regions, in the three slopes, expressed in (%)of dry plant material***

From the results, which are graphically presented (Figure 2), it can be seen that in the first and third slope the highest total nitrogen content was measured in the Tetovo region, and in the second slope and in all three slopes together in the Tetovo region, with the smallest content being measured in the Skopje region. Duncan's test for $p < 0.05$ and $p < 0.01$ showed no significant difference. The mean values of total nitrogen, at the level of the examined regions, in the three examined slopes separately and in the three slopes together, Duncan's test for $p < 0.05$ and $p < 0.01$ showed no significant difference.

**3.2. Proteins**

The results which are presented in (Figure 3), shows the values of mean protein, expressed as a percentage, at the examined locations in the Republic of North Macedonia, at the region level in all slopes. The highest measured

protein content, in all slopes separately and in all slopes together is in the Tetovo region, at the location Dzepchishte and the smallest in the Ovche Pole region, at the location Mustafino.

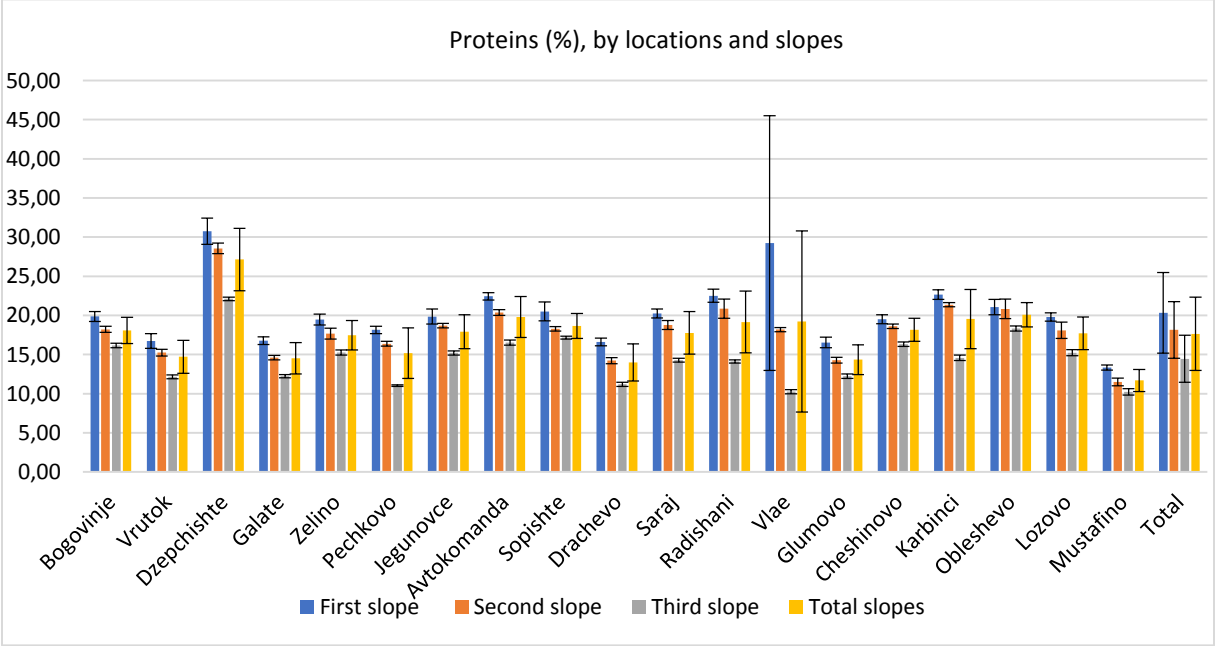


***Figure 3. The content of proteins at the examined locations from the three regions, in the three slopes, expressed in percentage (%) of dry plant material***

At figure 4 are presented the results for the mean protein values, on the region level, in the three slopes separately and in the three slopes together, with the Duncan test for $p < 0.05$ and $p < 0,01$ showed no significant difference.

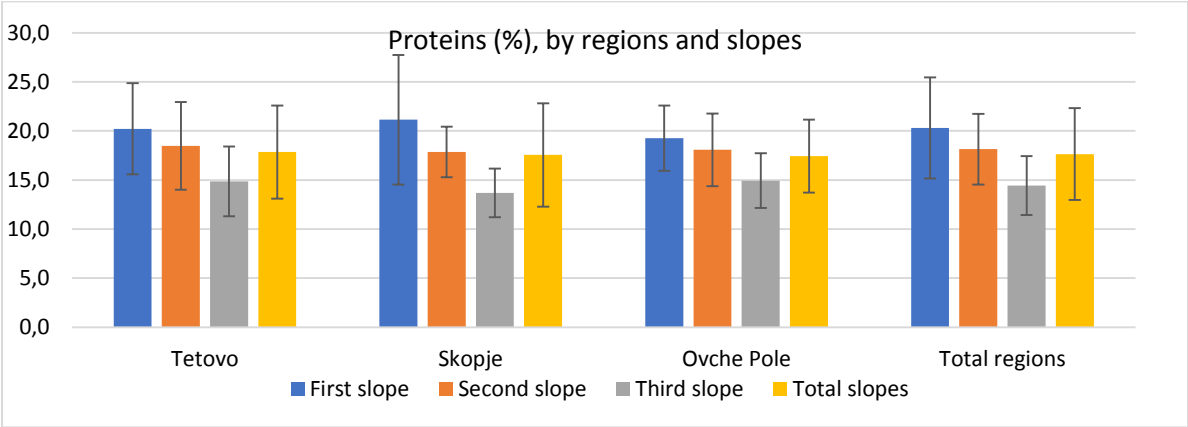


***Figure 4. Proteins content at the examined regions, in the three slopes, expressed in (%) of dry plant material***

**4. CONCLUSION**

In all slopes separately and in all slopes together, the highest content of total nitrogen was measured at the Tetovo region and the lowest content in the first and in the second slope was at Ovche Pole. In the third slope and in all slopes together, the lowest content was measured in the Skopje region. Significant differences can also be seen on the level of locations and regions. The mean values of total nitrogen, on the level of the examined regions, in the three examined slopes separately, as well as in the three slopes together, did not show any significant difference. Protein content is indicative of the fact that the Tetovo region has better conditions for protein production in alfalfa. The alfalfa has a high protein concentration in Tetovo region with favorable amino acid composition, resulting in a high biological value. In this sense, alfalfa is the dominant forage crop and active diet culture with high applicability to the bio-diet (Butleska Gjoroska, et al., 2018).

This paper provides a comprehensive analysis of nitrogen and protein composition of alfalfa grown in the Skopje, Tetovo and Ovche Pole region. This analysis is the first full and complete overview on the protein composition of alfalfa in the Republic of North Macedonia. The results will be great importance for further cultivation of this forage crop. Results has shown that the Tetovo region has a higher advantage compared to Skopje and Ovche Pole region in terms of nitrogen and protein content, which are crucial nutrients in forage crops.

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