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Determination of Saponins' and Coumarins' Content in Melliot Using the Method of NMR Spectroscopy

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Abstract

This article shows the results of determining the content of coumarins and saponins in melilot using the method of nuclear magnetic resonance (NMR) spectroscopy, including studying the influence of growth stimulants on the quantitative content of coumarins and saponins in the composition of melilot processed by the phases, using the method of 1H and 13C NMR spectroscopy. By the results of NMR spectroscopy, the possibility of qualitative and quantitative determination of the content of coumarin and saponin compounds isolated from the extracts treated with melilot growth stimulants has been assessed. The influence of three growth stimulants for plants, and their concentrations in the solution on the contents of coumarin compounds in the yellow melilot variety Kokshetau 10 has been studied. It has been shown that the used growth stimulants influence insignificantly coumarins' content in melilot, and consequently, quality of fodder based on it. In the studied ethanolic extracts, the most significant changes in the content of coumarin substances did not exceed 25%, compared to the reference sample that had not been treated with growth stimulator.

Keywords: Coumarins; Growth Stimulants, Melilot, NMR spectroscopy, Saponins.

INTRODUCTION

Melilot (Melilotus) is the herb capable of cultivation on unsuitable lands, where other herbs do not grow, and providing 18,000-20,000 kg/ha of green mass that is rich in protein and vitamins [1; 2]. Resolving this essential national economic task will require about two thousand tons of seeds of perennial grasses, including melilot. Currently, over 30% of arable lands in Northern Kazakhstan are waste lands or degraded grasslands that do not provide economic income. In the Kokshetau region, there are more than 303 thousand ha of arable land with the spring wheat grain yield below 500 kg/ha, which are subject to intensive meadow formation. Gradual meadow formation in the lands out of use is one of the ways of preserving the arable fund, which allows not only introducing the farmland into circulation, protecting it from degradation, but also additionally obtaining a lot of cheap fodder [3].

All parts of the melilot plant contain coumarin compounds, i.e., substances that are harmless to the organism of animals but have strong aroma [4; 5]. The formula of coumarin itself, which found in free form in small amounts in plants, is $C_9H_6O_2$. Most coumarin compounds are present in the form of coumarinic acid (glucoside). By losing water molecules, coumarinic acid turns back into the alkaline form, coumarin. Formation of coumarin compounds directly depends on the lighting conditions and the temperature. These factors influence the growth and development of plants and coumarin content and stimulate metabolism [6].

At the present stage of development of the analytical methods, the use of Nuclear Magnetic Resonance (NMR) opens new possibilities in the identification and determination of organic compounds. The technique of NMR spectroscopy is one of the most efficient methods of structural research. It allows obtaining information about the structure of molecules, about the nuclei present in the identified substance, and their quantity, and about their environment. One of the NMR methods, Proton Nuclear Magnetic Resonance (1H-NMR), is often the only one that allows for determining the structure of organic compounds. Along with 1H-NMR spectroscopy, 13C-NMR spectroscopy, mass and IR spectroscopy are used in experiments. Most often, the method of NMR spectroscopy is used for identifying nuclei of hydrogen (protons, 1H) and carbon 13C. One can also determine the content of other magnetically susceptible nuclei in the samples, such as19F, 31P, 17O, 15N, and 29Si [7].

This research was aimed at studying the influence of growth stimulants on the quantitative content of coumarins and saponins in the composition of melilot applied by the phases using the 1H and 13C NMR spectroscopy method.

The tasks of the research are as follows: determining the quantitative content of coumarins and saponins in melilot using the method of 1H and 13C NMR spectroscopy and studying the effect of growth stimulants on the fodder quality and the coumarin content.

MATERIALS AND METHODS

The object of the study is the yellow melilot variety Kokshetau 10, which is a promising variety in Northern Kazakhstan.

Yellow Kokshetau melilot 10 was selected at the North Kazakhstan Research Institute of Agriculture. Authors: Sagalbekov U. M., Onalov S. J., Sagalbekov E. U., Kusainova M. E. It is a complex synthetic hybrid population created by the polycross method from limited free pollination of biotypes and composition of varieties Alsheevsk, Kokshetau, Omsk precocious and Siberian 2. Breeding was aimed at the maximum yield of the vegetative mass about the growth vigor, tilling capacity, seed production, winter hardiness, drought resistance and quality of the fodder mass obtained from the plants. The variety belongs to yellow melilot (Melilotus officinalis L.) [8].

Field experiments were performed in 2015-2017 at the North Kazakhstan Scientific Research Institute LLC; these experiments were laid out in the framework of the project named "Development of High-Performance Moisture-Efficient "Green" Technologies in the Dry Lands Aimed at Increasing the Productivity of Fodder Crops, Intensification of Agriculture and Livestock breeding in Kazakhstan" in the Republic of Kazakhstan, Akmola region, Zerenda district, settlement Chaglinka.

The field experiments were laid out in 3 repetitions. The agricultural technology in the experiments was zoned. The area of experimental plots was 15 m2, plots' layout was randomized. The predecessor was fallow.

The experimental scheme included the following variants:

1 – control (water);
2 – grade B super Bio lignohumate;
3 – Hanse Plant Seedspor-C; and
4 – BM potassium lignohumate.

Sowing was performed in wide rows with 75 cm interrow spacing. The melilot seeding rate in wide sowing was 8.0 kg/ha. Seeds were treated with three growth stimulators: grade B super Bio lignohumate (2.5 ml/l), Hanse Plant Seedspor-C (1.0 ml/l), and BM potassium lignohumate (2.5 ml/l). The seeds were treated with growth stimulants 12 hours before sowing, followed by drying.

Laboratory analysis for determining the content of coumarins and saponins was performed at the Kokshetau State University named after Sh. Ualikhanov at the engineering laboratory of NMR–spectroscopy located in Kokshetau, Republic of Kazakhstan, Akmola region.

At the engineering laboratory of NMR–spectroscopy, the content of coumarin and saponin in the samples of melilot treated with growth stimulants in the following phases of development were studied: spring regrowth, flowering, and budding.

Analysis of the NMR Spectra of Samples 1-4 Scanned in D2O

NMR spectra 1H and 13C were scanned at a spectrometer JNN-Jeol ECA 400 (frequencies 399.78 and 100.53 MHz, respectively) using solvent D2O. Chemical shifts were measured about the signals of residual protons of deuterated solvent.

Regulations about the use of preparations:

Yellow Kokshetau melilot 10 was treated with growth stimulants in the following phases: spring regrowth, flowering, and budding.

Grade B super Bio lignohumate is a highly humic-fulvic product containing 90% of humic acids, of which the share of fulvic acid is 25-40%. It is this components' ratio that ensures excellent properties of the lignohumate used as a growth stimulant, an immunomodulatory agent, and an antistress agent. The "super Bio" modification is a combination of lignohumate and a biological growth regulator, which is characterized by high content of analogs to phytohormones, polyunsaturated fatty acids, phytoalexins of chelated forms of biogenic microelements, and is intended for treating leaves and roots. The consumption rate is 2.5 ml/l (Lignohumate).

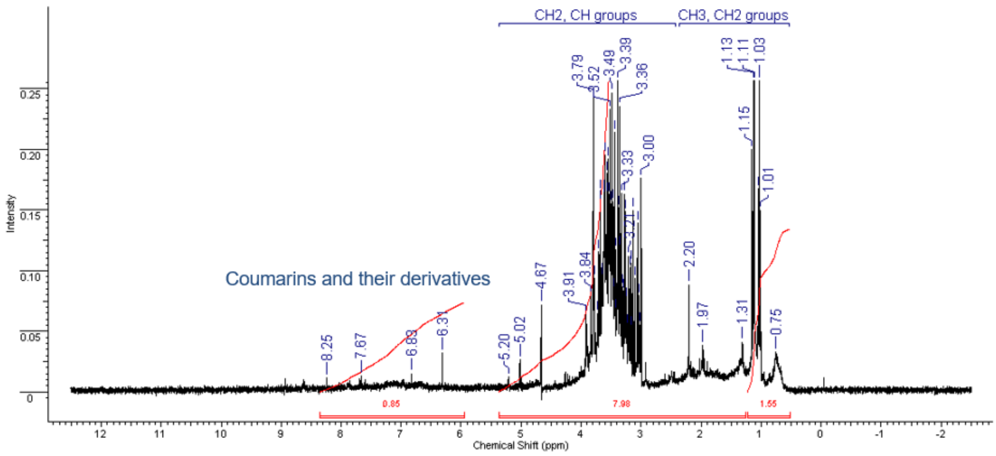
Hanse Plant Seedspor-C is a microbial product for soil and roots of cereals and legumes. The product contains highly efficient endomycorrhiza Glomus intraradices, which quickly settles in the roots of plants of many species, and optimally promotes plant growth, due to the increased consumption of water and nutrients. The fungus is supplemented by beneficial bacteria that stimulate rapid growth of the roots, and their complete mycorrhization. Besides, the bacteria bind nitrogen and release phosphorus, which is otherwise unavailable for plants. This results in increased productivity and saves chemical fertilizers. The consumption rate is 1.0 ml/l (http://www.hanseplant.com/).

Potassium grade BM lignohumate is a 20% aqueous solution with microelements. It is an efficient tool for the top dressing of all types of plants, for presowing treatment of seeds and root crops, and a humic supplement to mineral fertilizers. The solution contains 18% of salts of humic substances. The mass fractions of macro- and microelements are not less than: potassium - 9%, sulfur - 3% of the dry matter; and not more than: iron - 0.2%, manganese - 0.12%, copper - 0.12%, zinc - 0.12%, molybdenum - 0.015%, boron - 0.15%, cobalt - 0.12%. Calcium, silicon, magnesium are present. The concentration of hydrogen ion index (pH6%) is 8.5-10.0. The consumption rate is 2.5 ml/l (http://www.lignohumate.ru).

RESULTS

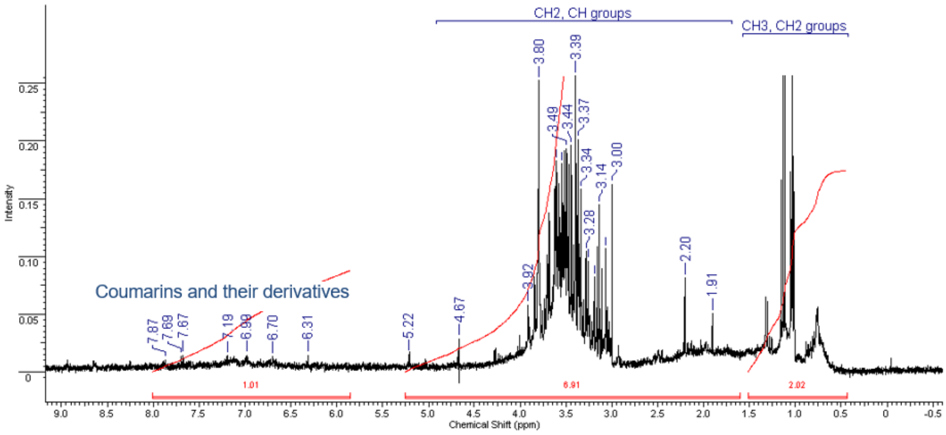
It is known that signals from the protons of coumarin and its derivatives should manifest themselves in the area of the weak field (6-8 ppm.), due to the peculiarities of the substance structure and the mutual influence of the atoms. Molecules of coumarins are dominated by aromatic fragments, which appear in the area shown above. In the proton spectrum of the studied compound of the reference sample in the indicated region, very few protons are observed. Their integral intensity is 0.85 H. This is the evidence of low content or the absence of coumarin derivatives in the sample.

Saponins and their numerous derivatives are triterpenoids and their derivatives; their molecules contain aliphatic methyl, methylene and methine groups and insignificant amounts of olefinic fragments. These protons appear in the NMR spectra in the area of 0.5-5.5 ppm. Based on the indicators shown in Figure 1, an overwhelming share of proton signals in the reference sample lie in this area. Their integral intensity is 9.53 H (Fig. 1).



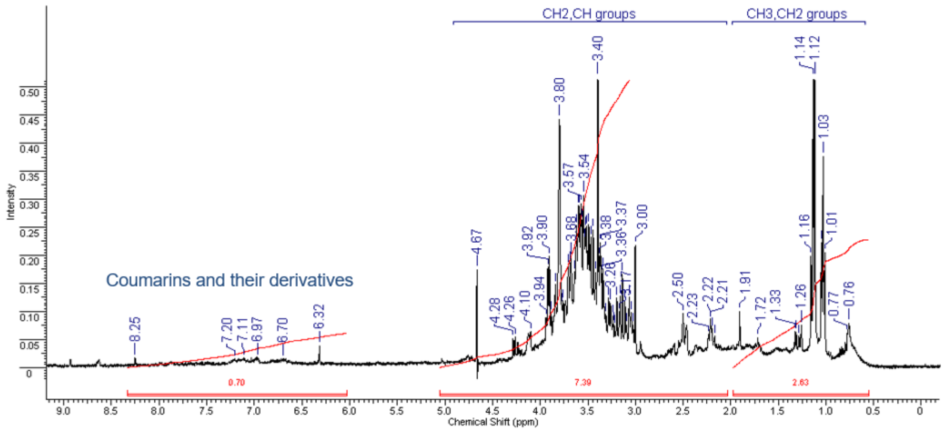
No.	(ppm)	Value	Absolute Value
1	[5.95 ..8.35]	0.852	1.31480e+1
2	[0.51 ..1.22]	1.547	2.38827e+1
3	[1.26 ..5.37]	7.983	1.23214e+2

Fig. 1. NMR proton spectrum of the reference sample.



No.	(ppm)	Value	Absolute Value
1	[5.85 .. 8.01]	1.014	1.39816e+1
2	[0.44 .. 1.52]	2.015	2.77751e+1
3	[1.60 .. 5.26]	6.908	9.52086e+1

Fig. 2. The proton NMR spectrum of the sample treated with grade B super Bio lignohumate.



No.	(ppm)	Value	Absolute Value
1	[6.02 .. 8.33]	0.700	1.40208e+1
2	[0.55 .. 1.97]	2.631	5.26932e+1
3	[2.03 .. 5.06]	7.393	1.48048e+2

Fig. 3. The proton spectrum of an NMR sample treated with Hanse Plant Seedspor-C.

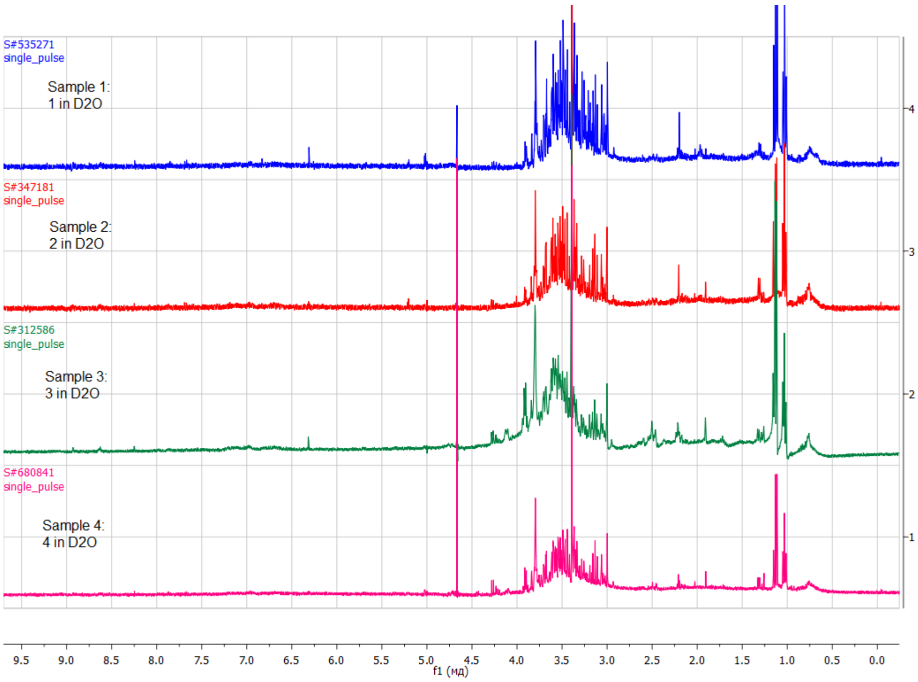


Fig. 4. Overlaying proton NMR spectra of 4 samples.

The quantity of coumarinic protons in the reference variant, compared to all protons in the extract, is 0.85 H: (0.85 H+7.98 H+1.55 H) = 0.0818 (8 and 18%).

In the sample where grade B super Bio lignohumate growth stimulant was used, the integral intensity of coumarinic protons was 1.01 H. The number of saponin and other protons in this sample was 8.93 H. The relative content of coumarinic protons in the sample was 1.01: (1.01+6.91+2.02) = 0.1016 (10.16%) (Fig. 2).

In the sample where Hanse Plant Seedspor-C was used as a growth stimulant, the integral intensity of coumarinic protons was 0.70 H. The number of saponins and other protons in this sample was 10.02 H. The relative content of coumarinic protons in the sample was 0.70: (0.70+7.39+2.63) = 0.0653 (6.53%) (Fig. 3).

In the sample where BM potassium lignohumate was used as growth stimulant, the integral intensity of coumarinic protons was 0.96 H. The number of saponins and other protons in this sample was 8.61 H. The relative content of coumarinic protons in the sample was 0.96: (0.96+6.53+2.08) = 0.1003 (10.03%) (Fig. 4).

DISCUSSION

Studying the effect of growth stimulants on the content of saponins and coumarins in melilot showed that in the variants treated with growth stimulants, and in the control variant, it did not change significantly.

The results of NMR spectroscopy allowed qualitative and quantitative determination of the content of coumarin and saponin compounds isolated from the extracts of melilot treated with growth stimulants. In spring regrowth, flowering and budding development phases, when melilot was sprayed with growth stimulants, the concentration of coumarin did not significantly change. In the reference sample, the content of coumarin protons was 8.18 proton integrals, the maximum content of coumarin protons (up 10.16 H) was observed in the sample where grade B super Bio lignohumate was used as a growth stimulant. The number of saponins and other protons in the reference sample was 9.53 H, and the sample with the use of grade B super Bio lignohumate had the lowest figure that amounted to 8.93 H.

In the variant with using the Hanse Plant Seedspor-C growth stimulant, the integral intensity of coumarin protons was 0.70 H, while in the sample with the BM potassium lignohumate growth stimulant it was 0.96 H. Accordingly, the number of saponin and other protons in the samples was 10.02 H and 8.61 H, respectively.

CONCLUSION

Thus, the obtained results showed that the growth regulators used for treating melilot in the spring regrowth, flowering, and budding stages do not influence the content of coumarins and saponins in the conditions of Northern Kazakhstan.

Given the fact that the content of coumarins and saponins in melilot does not considerably change, the authors suppose that it is expedient to continue research of melilot cultivation with the use of growth stimulants in various development stages.

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