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Original Article

Effect of fertilizers and growth stimulants on the content of coumarin and its derivatives in the vegetative mass of melilot

Efeito de fertilizantes e estimulantes de crescimento sobre o teor de cumarina e seus derivados na massa vegetativa do meliloto

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Abstract

The paper presents the results of determining the content of coumarin and its derivatives in the melilot using nuclear magnetic resonance spectroscopy, including the study of the effect of fertilizers on the quantitative content of coumarin and its derivatives in the composition of the melilot treated by phases using the method of ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. Based on the results of the nuclear magnetic resonance spectroscopy, the possibility of qualitative and quantitative determination of the content of coumarin and its derivatives isolated from extracts of fertilized melilot was evaluated. The effect of eight preparations on the plant and its concentration in solution on the content of coumarin substances in the Altynbas yellow melilot variety was studied. It was shown that the preparations have a slight effect on the coumarin content in the melilot and, accordingly, on the quality of feed based on it. In the control variant (variant 9), the coumarin proton content was 9.85%, and the maximum coumarin proton content was observed in the variant where BioEnergy was used as fertilizer and amounted to 22.44%.

Keywords: melilot, nuclear magnetic resonance spectroscopy, organic fertilizers, organomineral fertilizers, mineral fertilizers.

Resumo

O artigo apresenta os resultados da determinação do teor de cumarina e seus derivados no meliloto por meio da espectroscopia de ressonância magnética nuclear, incluindo o estudo do efeito dos fertilizantes no teor quantitativo de cumarina e seus derivados na composição do meliloto tratado por fases, utilizando o método de espectroscopia de ressonância magnética nuclear de 1H e 13C. Com base nos resultados da espectroscopia de ressonância magnética nuclear, foi avaliada a possibilidade de determinação qualitativa e quantitativa do teor de cumarina e seus derivados isolados de extratos de meliloto fertilizado. Foi estudado o efeito de oito preparados na planta e a sua concentração em solução no teor de substâncias cumarínicas na variedade de meliloto-amarelo Altynbas. Foi demonstrado que os preparados têm um ligeiro efeito no teor de cumarina do meliloto e, consequentemente, na qualidade dos alimentos à base dele. Na variante controle (variante 9), o teor de prótons de cumarina foi de 9,85%, e o teor máximo de prótons de cumarina foi de 22,44%, observado na variante em que a BioEnergy foi utilizada como fertilizante.

Palavras-chave: meliloto, espectroscopia de ressonância magnética nuclear, fertilizantes orgânicos, fertilizantes organominerais, fertilizantes minerais.

1. Introduction

Crop production is a branch of agriculture from which people receive most of the basic foodstuffs, animal feed, and raw materials for the main industries (Baibussenov et al., 2022; Kunanbayev et al., 2024; Salkhozhayeva et al., 2022).

Melilot is a crop that can be used for several purposes: food, green fertilizer, and honey plant. Two types of biennial melilot are cultivated in Kazakhstan: yellow melilot (*M. officinalis* Desz.) and white melilot (*M. albus* Medic). In recent years a new species has been introduced into culture, the dentated melilot (*M. dentatus* Pers), which is represented by the Saraychik variety of the selection of the Atyrau branch of the Southwestern Research Institute of Plant Production and Animal Husbandry (Zhumadilova et al., 2015). The seeds of the annual melilot were first imported by S.A. Tereshchenko from Russia and sown in the Ontustik Agricultural Park on an area of 16 ha in 2019.

Even infertile lands, including loose sands and heavy salt marsh clays, are suitable for the growth of this plant

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(Almanova et al., 2023). As an animal feed, melilot is not inferior in nutritional value to plants such as alfalfa, clover, and sainfoin, and in terms of protein content, it surpasses even corn (Bayazitova et al., 2023; Kozhanov et al., 2023). Scientists are working to assess the total unifying ability of the parent plants of the melilot based on leaf formation, feed productivity, and seed productivity (Bekimova et al., 2021). The study of the chemical composition of melilot varieties on saline soils is extremely important for preserving soil fertility and for its use in food for farm animals (Ivanova et al., 2022; Nugmanov et al., 2023).

Despite the high protein content and nutritional value, the widespread introduction of melilot into culture is hindered by several reasons (Adekiya et al., 2022). The most important of them is the ingrained opinion that it contains a large amount of an alkaloid harmful to animals, coumarin, which reduces the digestibility of animal feed. Coumarin is found in all parts of the plant in an amount from 0.03 to 1.5% per dry substance (Abdel-Baky et al., 2023). However, data on the toxicological effect of coumarin on animals are contradictory. Thus, Bokov et al. (1965) and Masalimov (1991) believe that including melilot in animals' diets improves their digestion by increasing pancreatic secretion (Chernova et al., 2019; Ejaz et al., 2023).

Currently, scientists are interested in studying the amount of coumarin in leaf and stem mass (Baidalin et al., 2017), its synthesis and biological assessment (Ibrahim et al., 2016), the use of coumarin derivatives in pharmacology (Arshad et al., 2014), its antimicrobial properties (Santos et al., 2023), and the effect of biological preparations on germination, growth, and development of perennial grasses (Kalin et al., 2023; Suraganov et al., 2018).

Melilot contains coumarin and its derivatives (0.4-0.9%: coumarin, dicumarol, dihydrocoumarin, glycoside melilotoside), flavonoids, melilotin, essential oil, polysaccharides, protein, saponins, purine derivatives, phenolic carboxylic acids, phenolic triterpene compounds, carbohydrate compounds, nitrogenous bases, amino acids, tannins, vitamin C, vitamin E, carotene, fat-like substances, and macro- and microelements.

Chemically, the substances contained in the melilot are used for various purposes, for example, the polysaccharides of the melilot restore the process of hematopoiesis in animals exposed to gamma rays and have an anti-inflammatory effect (Sychev, 2008). Melilot contains coumarin and its derivatives, including dicumarol with the properties of a strong anticoagulant. The plant has a pungent coumarin smell and a bitter taste.

Nuclear magnetic resonance (NMR) spectroscopy is a spectroscopy technique that is used to determine the unique structure of a compound. Due to this method, the carbon skeleton of an organic compound can be determined. Using this and other instrumental analysis methods, including infrared and mass spectroscopy, one can identify the entire structure of the molecule (Shvannikov, 2019).

Currently, such methods of NMR spectroscopy as ^{1}H-NMR spectroscopy and ^{13}C-NMR spectroscopy are widely used (Kachala et al., 2013).

The following research question follows: Will the amount of coumarin content in the melilot increase with the application of fertilizer?

The purpose of the study is to evaluate the effect of various types of fertilizers and growth stimulants on the content of coumarin and its derivatives in the melilot vegetative mass.

2. Materials and Methods

The object of the study was the Altynbas melilot variety. It was selected by breeders of the A.I. Baraev Scientific and Production Center of Grain Farming (Kazakhstan, Akmola region, Shortandinsky district). The variety was bred by E.I. Parsaev, T.M. Kobernitskaya, N.I. Filippova, G.V. Devyatkina, and G.N. Churkina.

The Altynbas yellow melilot variety has been included in the State Register of Breeding Achievements of the Republic of Kazakhstan since 2015 and is allowed to be used in production in the Pavlodar, Akmola, Karaganda, and North Kazakhstan regions. The variety is patented, and a patent of the Republic of Kazakhstan has been obtained.

Field experiments were conducted in 2022-2023 in Kokshetau Experimental Production Farm as part of the research on the project "Improving the technology of cultivating melilot for seeds according to the green principle with elements of organic farming in the Akmola region" within the framework of competition for grant financing of research by young scientists under the Zhas Galim project for 2022-2024 in the Republic of Kazakhstan, Akmola region, Zerendinsky district. We conducted this study in nine variants listed in Table 1.

The field experiments were established in 4-fold repetition. The agricultural technology in the experiments was zonal. The area of the experimental plot was 15 m^2, and the placement of plots was randomized. The preceding crop was black fallow.

The sowing method was wide-row, with 75 cm between rows. The seeding rate of melilot seeds with the wide-row method was 8.0 kg/ha. Eight preparations were used in seed treatment: Ammophos, BioEnergy, Isobion, Humate 7B, Gumi, BIOselitra, Fulvimax, and Start Up. These preparations were selected based on the availability of mineral properties suitable for organic farming. Seed treatment with growth stimulants was carried out 12 hours before sowing, followed by their drying.

Laboratory analysis for the content of coumarin and its derivatives was carried out in the NMR spectroscopy engineering laboratory of Kokshetau University named after Sh. Ualikhanov (Kazakhstan, Akmola region, Kokshetau).

In the NMR spectroscopy engineering laboratory, studies were carried out on samples of melilot of the first year of life, treated with preparations twice during the growing season, for the content of coumarin and its derivatives.

Analysis of NMR spectra of samples 1-9 taken in D_2O.

^{1}H and ^{13}C NRM spectra were taken using a JNN-ECA Jeol 400 spectrometer (frequency 399.78 and 100.53 MHz, respectively) using a D_2O solvent. Chemical shifts were measured relative to the signals of the residual protons of the deuterated solvent.

Table 1. Preparation options in the study.

Variant number	Variant name	Preparation description
1	Control	without the use of fertilizers
2	Ammophos	a complex nitrogen-phosphorus mineral fertilizer that is highly soluble in water. It contains phosphorus in high concentration but in an easily soluble form. It is available in the form of powder and granules of light gray or white color. This granular fertilizer is coated with a shell that prevents moisture absorption and caking but does not interfere with dissolution in water (Doronina and Ispolatov, 2023)
3	BioEnergy	a complex liquid fertilizer quickly absorbed by the root hairs of plants, providing optimal productivity, which guarantees balanced vegetative and generative development, increasing the overall metabolism in plants (Harnez Online Store of Farm Products, 2023a)
4	Isobion	an organomineral fertilizer designed to increase yields due to better fruit setting and bigger fruit size, improve the quality of marketable products, the survival rate of seedlings, the plant's ability to overcome various stresses, and the overwintering of perennial plants. A group of agrochemicals by chemical nature: a liquid organomineral fertilizer consisting of a mixture of amino acids and peptides (hydrolyzed protein) produced from leather production waste by alkaline hydrolysis followed by filtration and removal of insoluble calcium from the final product (Syngenta, 2023)
5	Humate 7B	a liquid concentrated organomineral fertilizer with a set of macro- and microelements (a mixture of potassium and sodium salts of humic acids, Cu, Zn, Mn, Co Fe, B) (Rosselhoscenter, 2023)
6	Gumi	mineral fertilizers used to stimulate the growth of garden and vegetable crops. This complex is a humic preparation since up to 60% of its mass consists of sodium salts. The remaining 40% are traditional mineral fertilizers (NPK), with additional trace elements. When developing this top dressing, a standard NPK complex was used as a basis, which has been used in industrial agriculture for decades (Masaleva, 2023)
7	BIOselitra	a growth stimulant that eliminates nitrogen deficiency, with a set of trace elements in chelated form with fruit-forming properties and fungicidal action in elevated concentrations (Biomeliorant, 2023.)
8	Fulvimax	an organomineral fertilizer with 40.8% organic substances, 3.4% N, organic soil improver in liquid suspension, contains 20% humic acids and 80% fulvic acids. It has an acidic character and a high ability to form and transfer other nutrients. The preparation can be applied separately or in a mixture with basic fertilizers or trace elements, through leaf dressing or by fertigation (Harnez Online Store of Farm Products, 2023b)
9	Start Up	an organomineral fertilizer, which contains 45% organic substances, 6% N, and 24% C (Agro-Kazakhstan, 2023)

Source: Compiled by the authors.

3. Results

In the ^{1}H NMR spectrum, a proton integral intensity of 19.56N was observed in the control variant in the aromatic region. This indicates the presence of coumarin derivatives in the studied variant.

In variant 2 with the Ammophos mineral fertilizer, the integral intensity of coumarin protons was 27.74 N (Figure 1). The number of saponin and other protons in the sample was 163.01 N. The relative content of coumarin protons in the sample was 14.54%.

In variant 3 with the BioEnergy complex fertilizer, the integral intensity of coumarin protons was 37.01 N (Figure 2). The number of protons of coumarin derivatives in the variant was 127.92 N. The relative content of coumarin protons was 22.44%.

In variant 4 with the Isobion organic fertilizer, the integral intensity of coumarin protons was 29.63 N (Figure 3). The number of saponin and other protons in this sample was 153.58 N. The relative content of coumarin protons in the sample was 16.18%.

In variant 5 with the Humate 7B organomineral fertilizer, the integral intensity of coumarin protons was 18.30 N (Figure 4). The number of protons of coumarin derivatives in this sample was 196.81 N. The relative content of coumarin protons in the sample was 8.51%.

In variant 6 with the Gumi mineral fertilizer, the integral intensity of coumarin protons was 23.12 N (Figure 5). The number of coumarin derivatives of protons in this sample was 146.71 N. The relative content of coumarin protons in the sample was 13.61%.

In variant 7 with the BIOselitra growth stimulator, the integral intensity of coumarin protons was 23.02 N (Figure 6). The number of coumarin derivatives in this sample was 166.06 N. The relative content of coumarin protons in the sample was 12.17%.

In variant 8 with the Fulvimax organomineral fertilizer, the integral intensity of coumarin protons was 22.28 N (Figure 7). The number of protons of coumarin derivatives in this sample was 167.87N. The relative content of coumarin protons in the sample was 11.72%.

In variant 9 with the Start Up organomineral fertilizer, the integral intensity of coumarin protons was 25.40 N (Figure 8). The number of protons of coumarin derivatives in the sample was 199.39 N. The relative content of coumarin protons in the sample was 11.30%.

For comparative analysis in the studied variants of protons of coumarin compounds and their derivatives, Figure 9 shows an overlay of ^{1}H NMR spectra of the experimental variants.

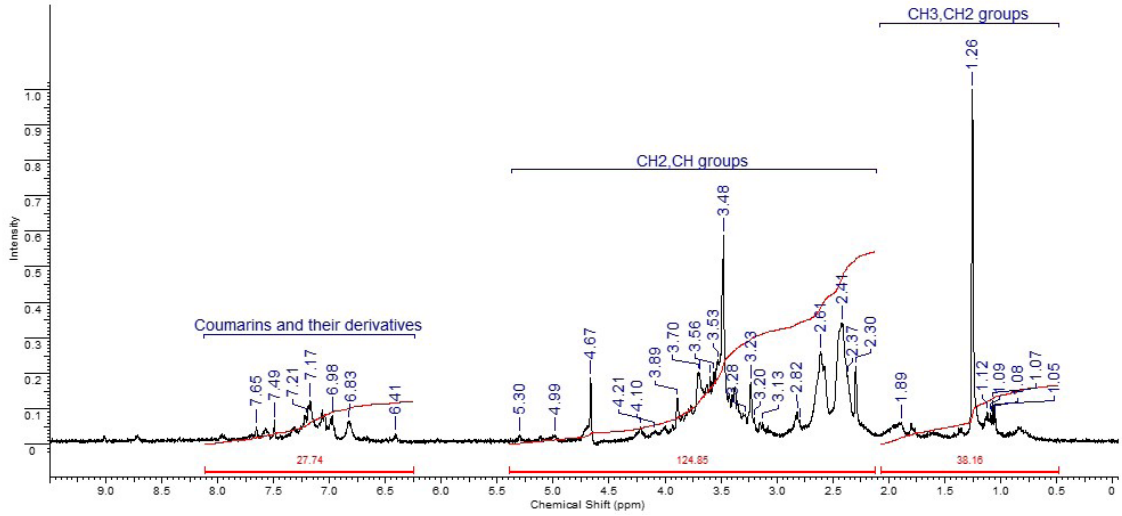

Figure 1. ^{1}H NMR spectrum with Ammophos.

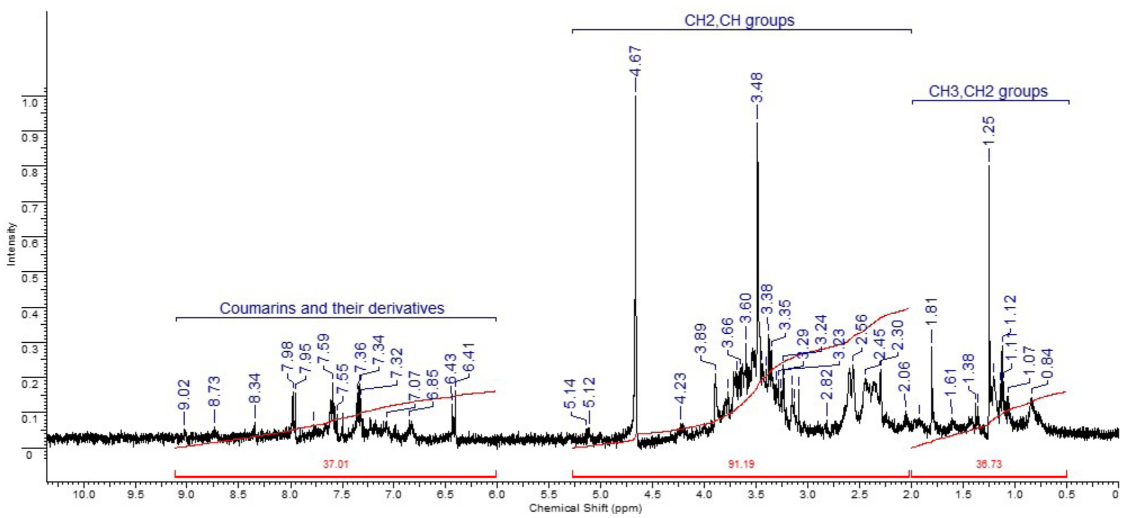

Figure 2. ^{1}H NMR spectrum with BioEnergy.

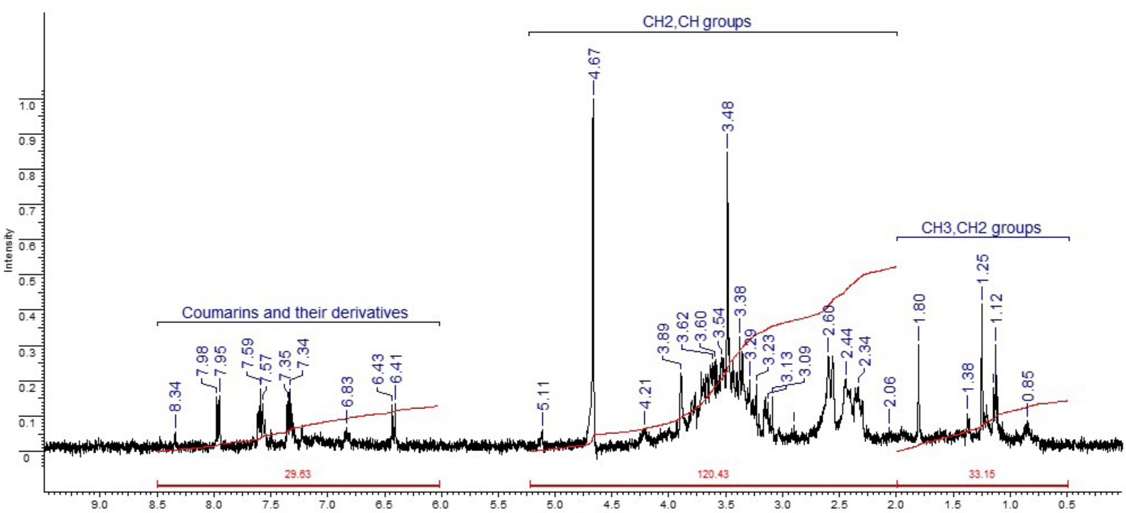

Figure 3. ^{1}H NMR spectrum with Isobion.

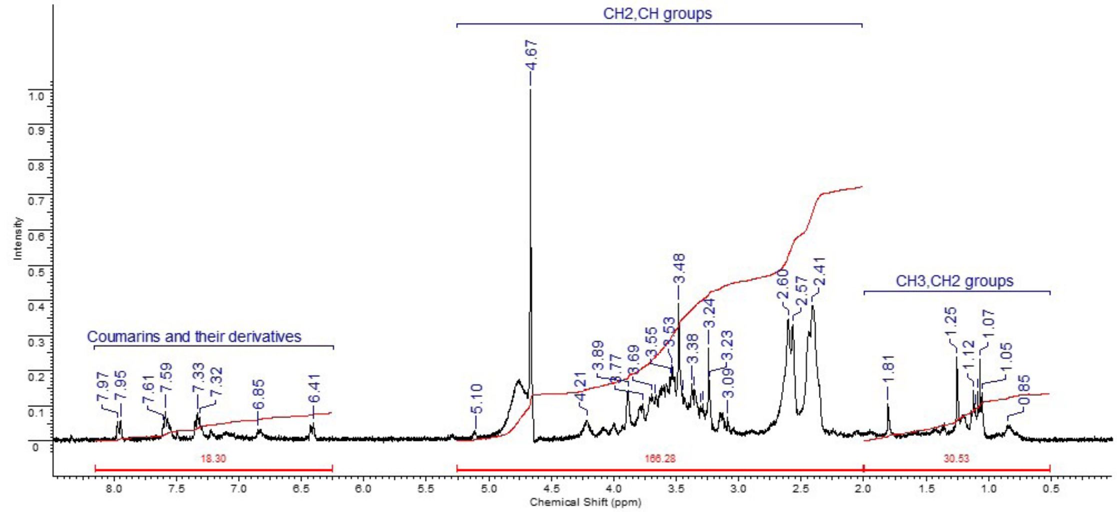

Figure 4. ^{1}H NMR spectrum with Humate 7B.

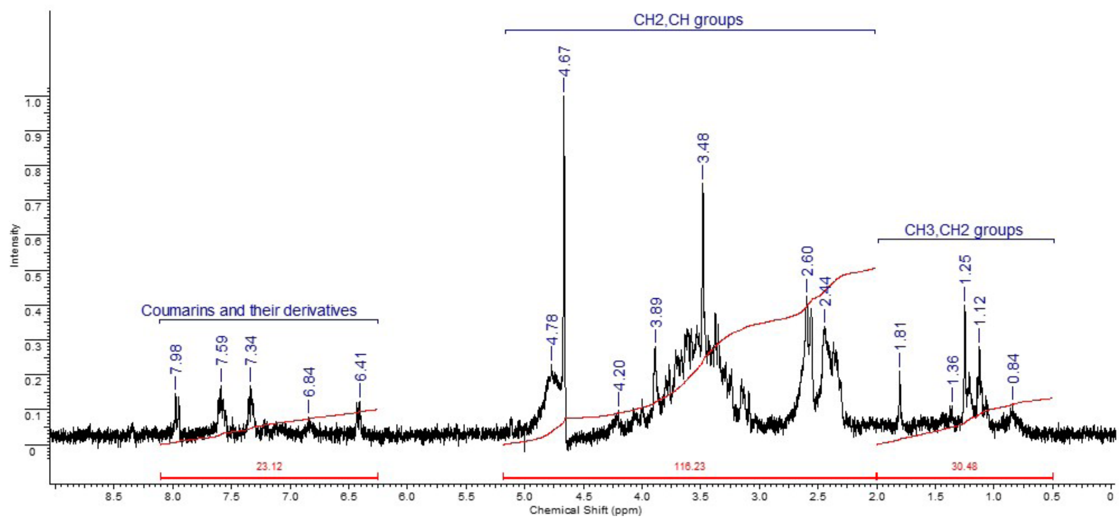

Figure 5. ^{1}H NMR spectrum with Gumi.

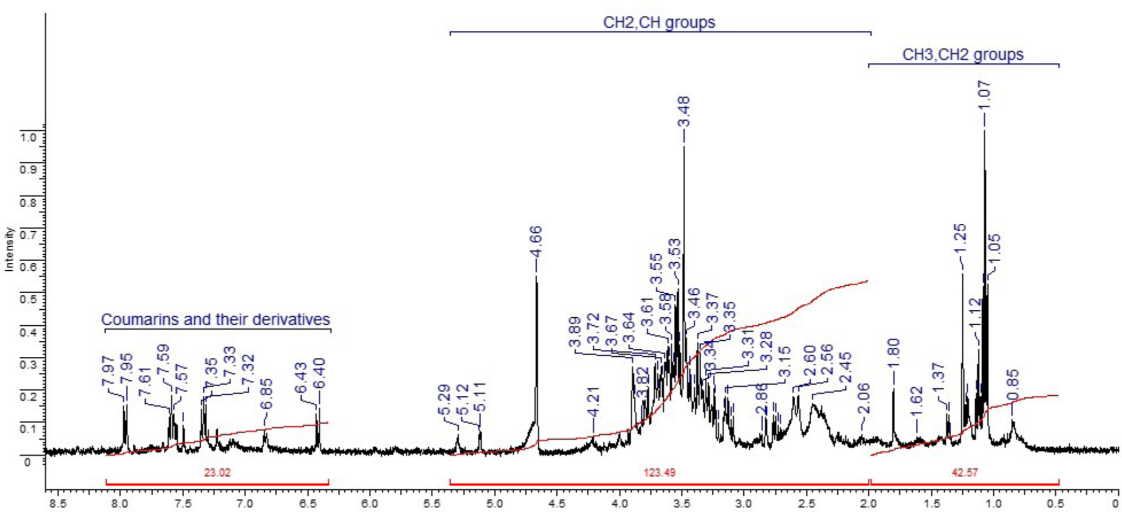

Figure 6. ^{1}H NMR spectrum with BIOselitra.

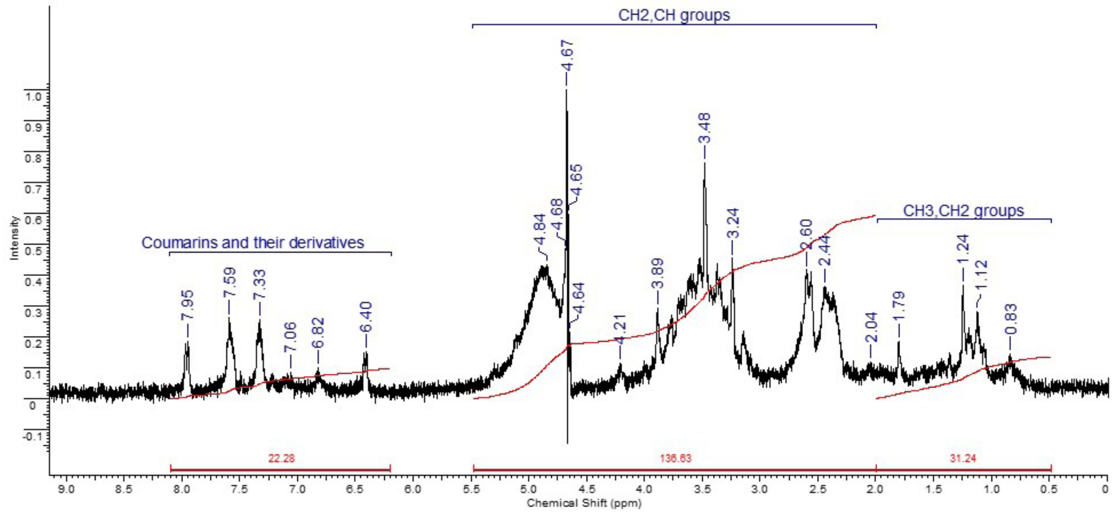

Figure 7. [1]H NMR spectrum with Fulvimax.

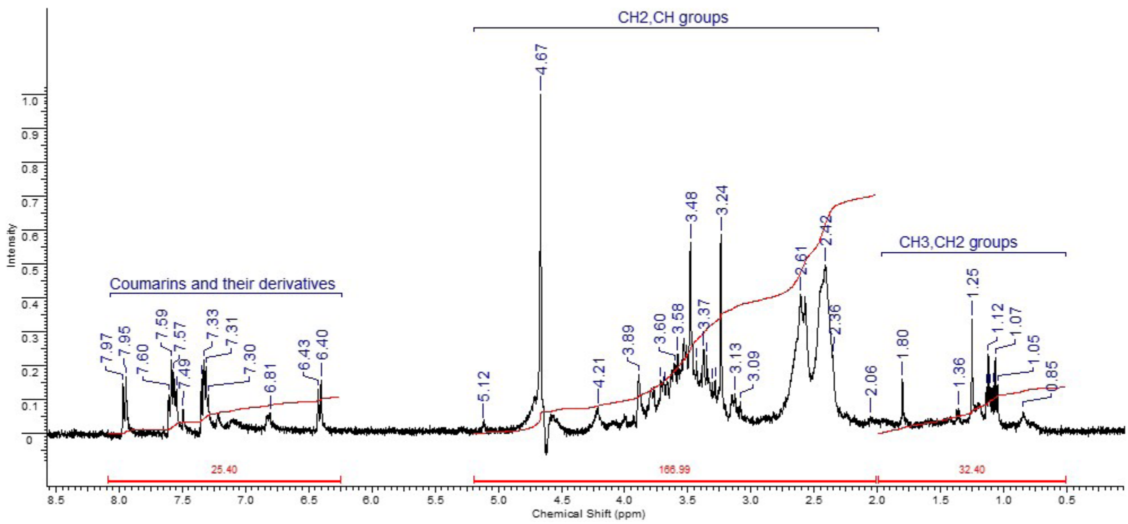

Figure 8. [1]H NMR spectrum with Start Up.

A visual examination of the overlayed [1]H NMR spectra of the experiment variants shows that in the variants, the relative content of coumarin protons and their derivatives is approximately the same. Moreover, in all variants, the content of protons and their derivatives exceeds the number of coumarins by an order of magnitude, except for the variant with BioEnergy (here, the coumarin proton content is 22.44%) and Isobion (here, the coumarin proton content equals 16.18%). In the remaining variants, the coumarin proton content ranges from 8.51% (Humate 7B) to 14.54% (Ammophos).

4. Discussion

The [1]H and [13]C NMR spectra were taken on a JNM-ECA Jeol 400 spectrometer (frequency 399.78 and 100.53 MHz, respectively) using D2O solvent in 5 mm wide ampoules at 250 °C. Chemical shifts were measured relative to the signals of the residual protons of the deuterated solvent.

Coumarin compounds are widespread and establishing their content in grass is an important task. Today, the most effective method for determining coumarin in vegetation is the NMR spectroscopy method (Kafarov et al., 2023; Lapshin et al., 2023; Nobel Nawab et al., 2023). Thus, in (Nobel Nawab et al., 2023), the spectra of more than 100 natural coumarins were studied and analyzed using [1]H NMR spectroscopy. The spectra of 209 natural and synthetic coumarins were studied using [13]C NMR spectroscopy (Kafarov et al., 2023). Therefore, the NMR spectroscopy method was chosen by us to analyze the content of coumarin and its derivatives in samples of melilot obtained using various organic fertilizers and plant growth stimulants. To determine the content of coumarin and its derivatives in a sample of melilot, it is divided into test parts, crushed, and dissolved in an appropriate

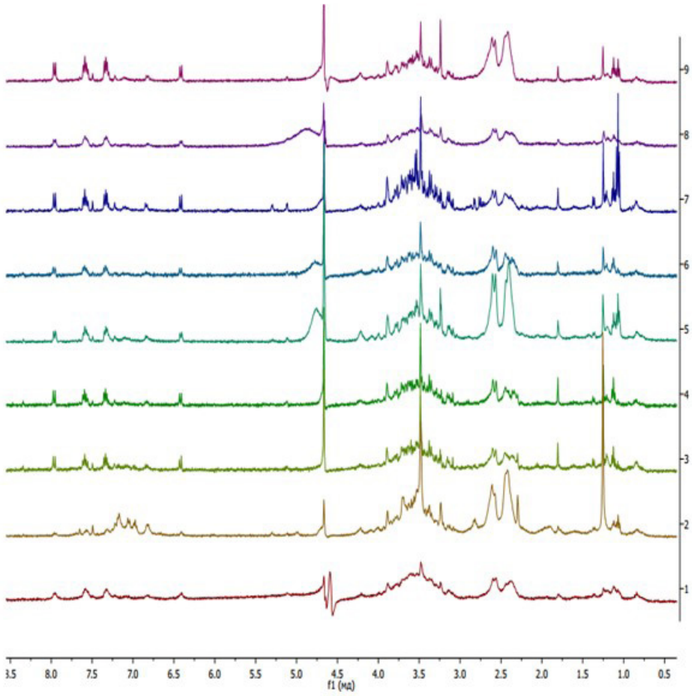

Figure 9. Overlay of ¹H NMR spectra.

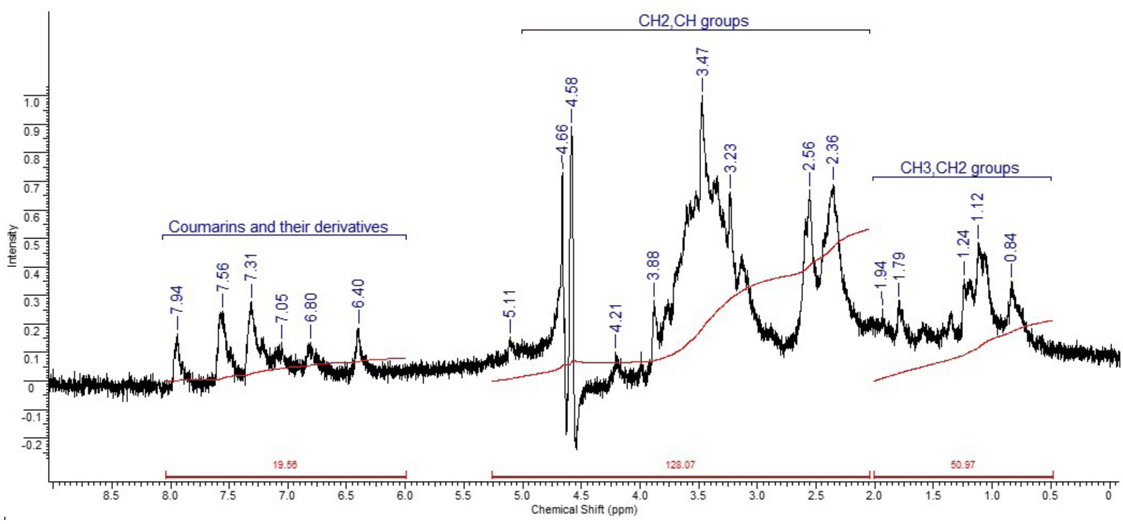

Figure 10. ¹H NMR spectrum in the control variant.

solvent, and then the extracted component is isolated. The dry isolated component is dissolved in deuterated water and the ¹H and ¹³C NMR spectra are taken. Since coumarin and its derivatives contain fragments of unsaturated aromatic lactones in their composition, the NMR signals of ¹H protons of coumarin and its derivatives should be manifested at 6-9 ppm.

The group of ¹H NMR signals in the range 0.5-5.5 parts per million (ppm) belong to methyl, methylene, methine, and olefin protons, which are found in triterpenoids and their derivatives. They are often found in saponins. The integral proton intensity of these fragments is 179.04 N (Figure 10). The relative content of coumarin protons in the control variant is 9.85%.

The results of NMR spectroscopy made it possible to evaluate the qualitative and quantitative content of coumarin compounds and their derivatives isolated from extracts of melilot treated with growth stimulants.

In melilot sprayed with preparations, the concentration of coumarin did not change significantly. It was determined that in the control variant, the coumarin proton content was 9.85%, and the maximum coumarin proton content was observed in the variant where BioEnergy was used as fertilizer (22.44%).

Based on the obtained results, it can be suggested that most of the preparations used for the treatment of melilot have a slight effect on the quality of feed and the coumarin content.

5. Conclusions

Based on the results of the study, we concluded that the addition of fertilizers to the soil has a slight effect on the content of coumarin protons and feed quality: the highest coumarin content was detected with BioEnergy and amounted to 22.44%. It should be borne in mind that the results of the study were limited by the specifics of the region and the conditions of growing melilot.

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