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# DRIFT and UV-VIS spectroscopic characterization of humic substances in grasslands after organic and mineral fertilization

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**Abstract**

Changes in the chemical composition and stability of humic substances after soil amendment with mineral and organic materials were studied. The study site, a Gleyic Fluvisol (Jaroměřice locality, Pardubice Region, Czech Republic), was under permanent grassland managed intensively (four cuts per year) and fertilised as follows: manure and slurry at a rate of 2 livestock units = 120 kg N per ha. The fertilization treatments included: C – control (no fertilization); FYM – farmyard manure (30.0 t ha$^{-1}$); CS – cattle slurry (29.0 t ha$^{-1}$); DIG – digestate (29.0 t ha$^{-1}$); NPK – mineral fertilizer (120–30–60 kg ha$^{-1}$). The study aimed to evaluate changes in the structural composition and stability of humic substances during the period 2022–2024, with the goal of providing a knowledge base for farmers to develop best practices for maintaining soil carbon stocks under intensive agricultural conditions. Humic substances (humic and fulvic acids) were extracted with a mixture of 0.1 M NaOH and 0.1 M $Na_4P_2O_7$ /1:1, w/w. Further, humic acids (HA) were isolated according to the standard IHSS method and characterized using UV-VIS and DRIFT spectroscopy. Calculated indices showed higher HA stability and wettability after FYM, DIG, and CS application in comparison with NPK and control. The differences were found in the content of labile aliphatic hydrophobic groups (at 3000–2800 cm$^{-1}$), aromatic stable and resistant C=C groups (at 1660–1580 cm$^{-1}$), and hydrophilic amido-, carboxylic-, keto-groups (at 1740–1600 cm$^{-1}$). The amount of hydrophilic resistant groups at 1660–1580 cm$^{-1}$ was comparable at all sites. DRIFT and UV-VIS spectral methods were proposed as rapid tools for assessing humic substance quality instead of the laborious and time-consuming classical fractionation method.

## 1. Introduction

Soil and the soil environment are paid considerable attention, both in terms of their quality, sustainable management, increasing carbon sequestration capacity, and general improvement of soil health (Borůvka et al., 2022; Hendricks et al., 2022; Kochiieru et al., 2022). Soil health is defined as the ability of (living) soils to function within natural or managed ecosystems, maintain plant productivity, and improve or maintain water quality and support plant and animal health (Doran et al., 1996, 2002; Lehmann et al., 2020; Wood and Blankinship, 2022; Pavlů et al., 2021a, 2021b, 2022; Lichtenberg, 2024). Soil health is defined by indicators (measurable properties) of the soil or vegetation that provide data on the soil environment and its function. Authors also mentioned that selected indicators are aimed at assessing productivity or ecosystem services (e.g. chemical, physical, biological properties of soil, including various processes, toxicity and human health effects). Most of the soil ecosystem services depend on the amount and quality of soil organic matter (SOM). It is a key factor of soil productivity, which affects binding of water and nutrients, and all-over soil chemical, physical and biological processes (e.g. nutrient cycles, temperature and water regimes, soil reaction, biological activity, aggregability, consistency, hydrophobicity, hydrophilicity, etc.) (Cornu et al., 2023; Pavlů et al., 2021a, 2021b). As quoted by Thai (2022) more hydrophobic character refers to higher SOM stability and protection against microbial decomposition. They argued that quality of organic materials directly affects humic substances activity and formation of organo-mineral complexes, which is important for stability of the whole agro-ecosystem. Monitoring of humic substances quality has a long history, and many controversial theories were proposed. Some authors (Lehmann and Kleber, 2015, 2019) rejected SOM quality assessment based on their solubility and fractionation described by Stevenson (1994) or Piccolo (2002). However, others are improving and developing the methods of SOM characterization after alkali extraction

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from soil (Jamroz et al., 2022; Wang et al., 2023). Recently, Rabot et al. (2024) suggested evaluating organo-mineral interactions in soil according to C/clay ratio. The ratio is also frequently used as an indicator of soil structural quality and the degree of soil organic carbon protection. Pavlů et al. (2021a, 2021b) documented that C/clay ratio is more a criterion for assessing soil degradation due to carbon loss then SOM quality. Trying to solve this problem indirect spectroscopic methods and technics are applied. Spectral technics are supposed to be fast, harmless, and relatively cheap. The one of most applied technics is infrared spectroscopy, which allows to determine different functional groups, stability, wettability and hydrophobicity of SOM (Leue et al., 2010; Demyan et al., 2012; Gholizadeh et al., 2018 and 2021. The mid-infrared (MIR) spectral region, DRIFT and FTIR techniques are highly recommended for SOM quality evaluation. Lei et al. (2023) showed these methods also provide information on the presence of polysaccharides, aliphatic and aromatic components, and water affinity. Furthermore, mineral fractions (e.g. quartz or carbonates) can be evaluated (Madejová, 2003; Pavlů et al., 2023a, 2023b). According to Thabit et al. (2024) a corrected spectral curve in MIR spectral region can be used for total organic carbon monitoring. A disadvantage of given methods is the spectral overlap of bands in the mineral soil samples, which caused some difficulties with results interpretation (Pavlů et al., 2021b). This is the main reason why different fractions of humic substances are isolated from soil (e.g. humic acids, fulvic acids, humins). The wide range of spectral techniques brings a lot of data, and there are broad databases of SOM properties in different arable soils (Bispo et al., 2017; Pavlů et al., 2023a,b; Nazaries et al., 2021; Feszterová et al., 2024). However, there is a gap of knowledge of SOM quality in grasslands, which are significant carbon sinks in ecosystems. Stationary small plot field experiment at Jevíčko was established in 2004. There was a need to focus on SOM quality and productivity increasing on grassland. Calculated spectral indexes can help not only to evaluate soil quality but also support the quality of agrotechnical measures in production. It is also important to get an answer to whether the SOM quality/stability affects the soil environment.

The research aims at detailed SOM characterization after amending grassland soil with organic and mineral fertilizers. Changes in quantity, stability, hydrophobicity, and chemical composition were assessed by classical fractionations methods and using UV-VIS and DRIFT spectroscopy. The further goal was to fill the knowledge gap concerning humic substances in grassland ecosystem. SOM characterization can support the appropriate management practices to increase SOM content and enhance soil health. The next goal was to highlight the advantages of spectroscopic methods, which offer faster and more detailed SOM characterization compared to the classical fractionation method.

## 2. Materials and methods

The long-term field was carried out at the Grassland Research Stationary Jevíčko (Czech Agrifood Research Centre / CARC/). The research was conducted in a long-term experiment in the form of precise small plots on permanent grassland (location: 49.6282881N, 16.7317036E; CARC, Jevíčko, Pardubice district, Czech Republic) established in 2004 at an altitude of 342 m above sea level, with an average annual temperature of 8.4°C and an average annual precipitation of 558 mm (Station Jevíčko /1991-2020/ CHMI Ostrava) (Fig. 1). The area is a part of the Boskovice furrow, which consists mainly of claystones (flakes) and red sandstones (Culek et al., 2005). Botanical composition of the permanent grassland (establishment of the experiment): meadow vegetation – oatgrass (*Arrhenatheretum*), dominant grass species – false oatgrass (*Arrhenatherum elatius* L.), cock's-foot (*Dactylis glomerata* L.), meadow foxtail (*Alopecurus pratensis* L.), Kentucky bluegrass (*Poa pratensis* L.), and red fescue (*Festuca rubra* L.) (Menšík and Nerušil, 2019). An investigation was conducted into the properties of soil under intensive grassland management: intensive /4 cuts/year/ (1st cut on 15 May, further cuts after 45 days) and levels of fertilization according to the cattle stocking rate (1 livestock unit = 60 kg N $ha^{-1}$): intensive /4 cuts/year/: 2 livestock units per ha (120 kg N). The precise small grassland plots were approximately 1.25 m x 8 m = 10 $m^2$ and they were established in four replications. The study variants were as follows: (1) control without fertilization (Control); (2) mineral fertilizers (NPK); (3) manure + slurry (FYM), (4) cattle slurry (CS); (5) digestate (DIG). The application doses are listed in Table 1. Soil sampling was done at a depth of 0–10 cm, in 5 variants and 4 repetitions during the period spring 2022 – 2024. Soil was classified according to Němeček et al (2011) as Gleyic Fluvisol. Selected soil properties are given in Table 2. Soil reaction was determined by the potentiometric method. Soil organic carbon content (SOC) was determined by oxidimetric titration method according to Nelson and Sommers (1982). Wet oxidation includes using potassium dichromate ($K_2Cr_2O_7$) and 96% sulphuric acid. Titration by 0.5 M ammonium iron(II) sulphate (Mohr's salt) was done by digital byrete Schott SI Analytics TITRONIC TZ3230 (Thermal Scientific, Inc. Mansfield, Texas, USA). Humus fractional composition was determined according to Kononova-Belchikova method (1963) as follows: 5 g of air-dried soil sample, sieved at the mesh size of 1mm and extracted by the mixture (1:1, 0.1 M NaOH + 0.1 M $Na_4P_2O_7$) for 24 h. The sediment was separated by centrifugation at 2800 g for 10 min, washed with the mixture, and centrifuge again. Two individual washings were unified with the original supernatant, acidified with concentrated $H_2SO_4$ to pH 1.5. HA were allowed to precipitate overnight. The sum of HS, HA and FA were determined by the titrimetric method in aliquot volumes. HA isolation was made as follows: 100 g of the air-dried soil sample, sieved at the mesh size of 1mm, washed by 10% HCl and stirred for 1–2 hours (decalcination process). After the negative reaction for $CO_2$ (detected by seeing no bubbles) the rest of the soil solution was washed by 0.05 M HCl. After the negative reaction for $Ca^{2+}$ (detected by ammonium oxalate), the rest of the soil solution was washed with distilled water. After the negative reaction for Cl-(detected by $AgNO_3$), the soil rest was shaken in 0.1 M NaOH for 7–8 hours. The solution was precipitated overnight and centrifuged 15 minutes at 5000 rpm. The dark-brown HS mixture was precipitated by concentrated HCl to pH=1. After coagulation HA samples were decanted, washed several times, extensively purified by 0.5%

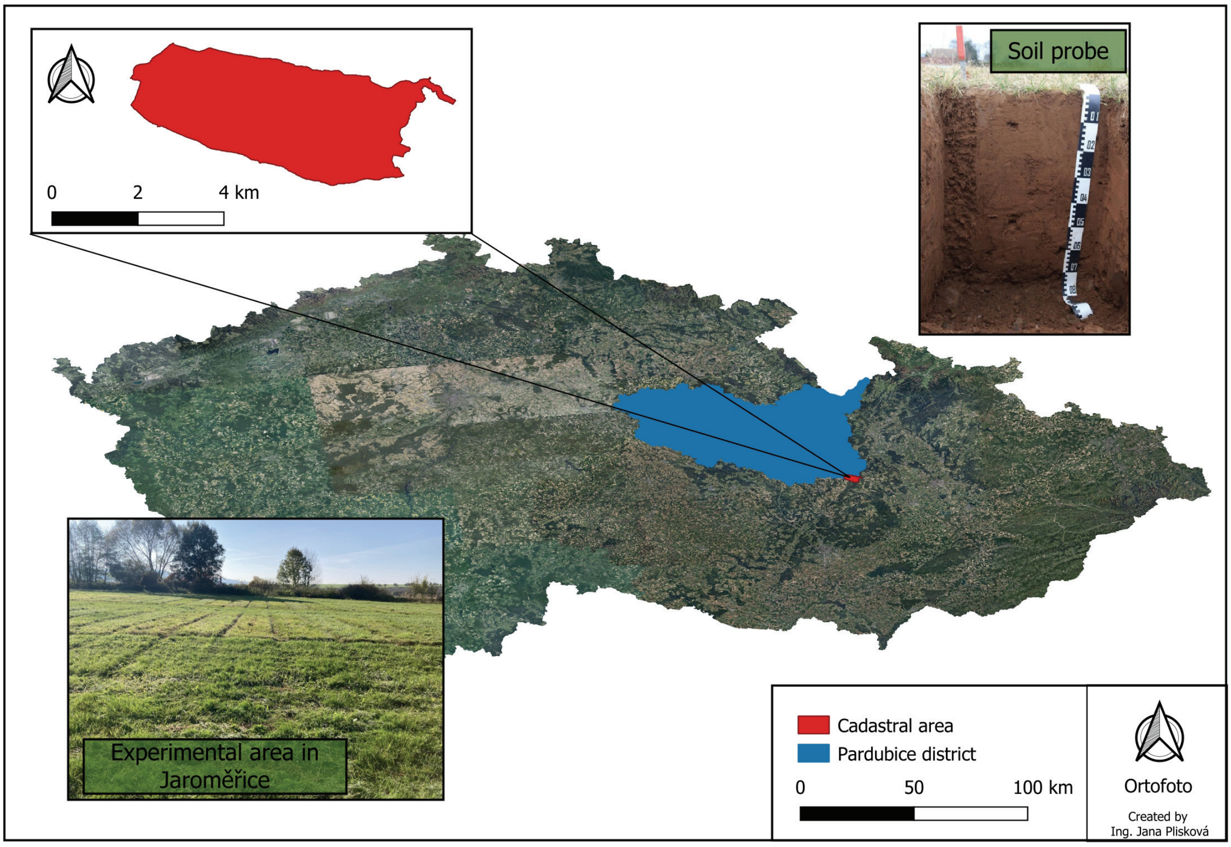



**Fig. 1.** Map of the studied area – locality Jaroměřice (the Czech Republic)

**Table 1**

| Variants | Mineral fertilisation | | | | | | Manure + Slurry * | | Cattle Slurry/Digestate* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | N | | | | P | K | | | | |
| | kg.ha$^{-1}$ of nutrients | | | | | | t.ha$^{-1}$ | | | |
| | Spring | after I. cut | after II. cut | after III. cut | spring | spring | autumn | after I. Cut | spring | after I. Cut |
| Control | – | – | – | – | – | – | – | – | – | – |
| NPK | 40 | 30 | 30 | 20 | 30 | 60 | – | – | – | – |
| FYM | – | – | – | 50 | | | 21,9 | 8,2 | – | – |
| CS | – | – | – | – | – | – | – | – | 14,5 | 14,5 |
| DIG | – | – | – | – | – | – | – | – | 14,5 | 14,5 |

* Explanations: *FYM – manure + slurry, CS – cattle slurry, DIG – digestate, NPK – mineral fertilizers;* actual fertiliser rates were based on the nutrient content of the fertiliser in a given year. The model load of TTP was established according to the joint methodology of HBLFA Raumberg-Gumpenstein and VÚRV, v.v.i. (Komárek et al. 2005).

**Table 2**

| Horizon | Depth (cm) | Texture | pH/KCl* | pH/$H_2O$ | $N_{tot}$ (%) | SOC (%) | CEC (cmol(+) kg$^{-1}$) |
|---|---|---|---|---|---|---|---|
| Ad | 0-10 | SL | 5.29 | 5.96 | 0.22 | 1.87 | 21.92 |
| Bt | 10-30 | SL | 5.32 | 6.25 | ND | 0.85 | 15.50 |

* Explanations: pH/KCl – exchangeable soil reaction; pH/$H_2O$ – active soil reaction; Ntot – total nitrogen content; SOC – soil organic carbon content; CEC – cation exchange capacity; SL – sandy loam texture soil; ND – not determined.

mixture HCl + HF, and dialyzed against distilled water until chloride-free, and freeze-dried. Detailed description of all mentioned methods is given by Stevenson (1994), Hayes (1985), Piccolo (2002), Pospíšilová et al. (2015 and 2016), and Plisková et al. (2023). Statistical analysis, including ANOVA, was conducted using the program Statistica 14 (TIBCO Software Inc., Palo Alto, USA, 2018).

UV-VIS spectra were measured in the mixture (1:1, 0.1 M NaOH + 0.1M $Na_4P_2O_7$). UV-VIS spectra were recorded within 300 $cm^{-1}$ to 700 $cm^{-1}$ using a Varian Cary 50 spectrometer (Varian Mulgrave, Victoria, Australia). Calculated indexes in UV-VIS (A446/A665) were calculated according to Kumada (1987) and Voltr et al. (2021). The results were statistically evaluated (ANOVA /Tuckey's test/).

For DRIFT analysis, the soil and HA sample (100 mg) were mixed with 300 mg KBr (FTIR grade 99%, Aldrich, Germany) and ground in an agate mortar. All spectra were measured using Thermo Nicolet Avatar 320 FT-IR spectrometer (Nicolet, Madison, WI, USA) operating with Smart Diffuse Reflectance accessory. Three DRIFT spectra (absorption mode, KBr background, 256 scans, data spacing 1.929 $cm^{-1}$) were collected with each HAs and soil sample. The spectra were analysed at four absorption bands that indicate the aliphatic hydrophobic CH-groups (denoted as A), aromatic C=C groups, and hydrophilic CO-groups (denoted as B, C, and D) (Demyan et al., 2012). The C-H bands occurred at 3000–2800 $cm^{-1}$ signal area and COO-, C=C and C-O bands occurred at 1660–1580, 1546–1520 and 1740–1600 $cm^{-1}$ signal area. Spectra were integrated by the spectrometer software (Omnic, version 6a). Humification degree (B+C)/A) and hydrophobicity index (D/A) were calculated (Ellerbrock et al., 2005; Demyan et al., 2012). The data were processed by analysis of variance followed by the Tukey HSD test that evaluates the significance of differences between the variants.

## 3. Results

The SOM fractionation results obtained from three years of observation (spring 2022, spring 2023, and spring 2024) on the grassland are listed in Fig. 2–6. As it is shown, the humus content was highest after farmyard application and varied from 3.12 to 3.52%. On the other hand, the mineral fertilizers (NPK) had the lowest humus content (2.17–2.75%). In spring 2022 statistically significant differences were found between control, manure + slurry (FYM), cattle slurry (CS), and digestate (DIG) variants. Also, the differences in humus content between C, CS and NPK variants were statistically significant (Fig. 2). In spring 2023, the same results were obtained, and there were statistically significant differences between C, FYM, CS and DIG variants. In spring 2024, a slight increase in humus on all variants was observed, except for the FYM variant. This might be caused by heavy rainfalls in this region and changes in soil biological activity. The measured values of humus content (2.2–3.5%) in all studied variants can be evaluated as medium or high (3.5% for FYM variant). Lower values were typical for mineral fertilizing and higher for FYM application. Evaluation was done according to Voltr et al. (2021). Differences in humus content between FYM and NPK variants were statistically significant. Similar results after amending grassland soils by organic fertilizers were published by Rambaut et al. (2022). The content of humic substances (HS) determined after alkali extraction and short fractionation is given in Fig. 3. The highest HS amount was determined on the FYM variant and varied between 0.52–0.83%. Statistically significant differences were found between FYM and all other variants (DIG, CS, NPK). Differences between the control (untreated site) and the FYM variant were significantly different. The dynamic of humic substances follows the dynamic of humus (Fig. 2 and 3). It was concluded that FYM and slurry application resulted in the highest amount of SOM.

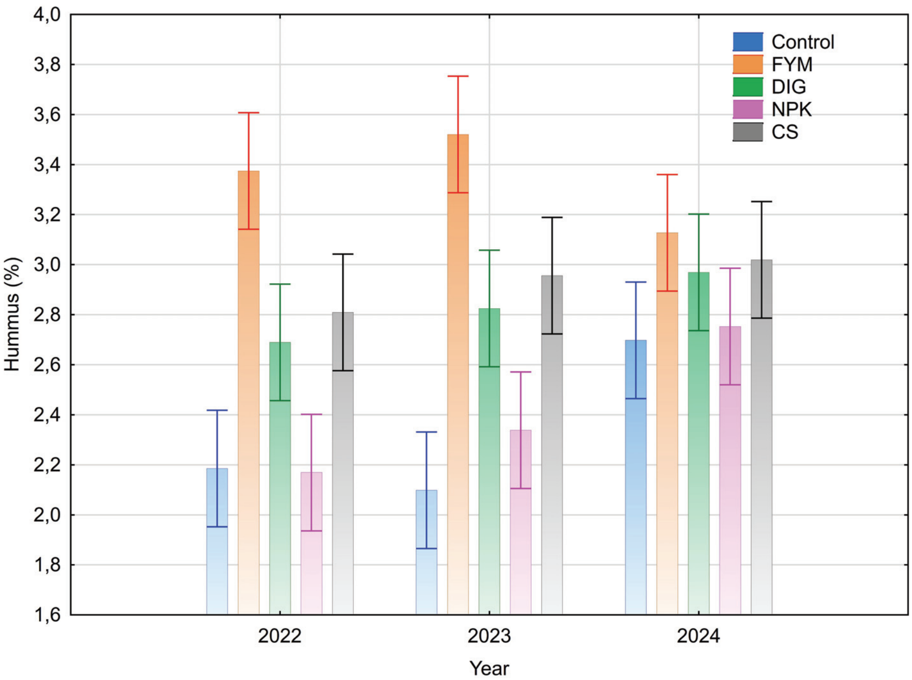
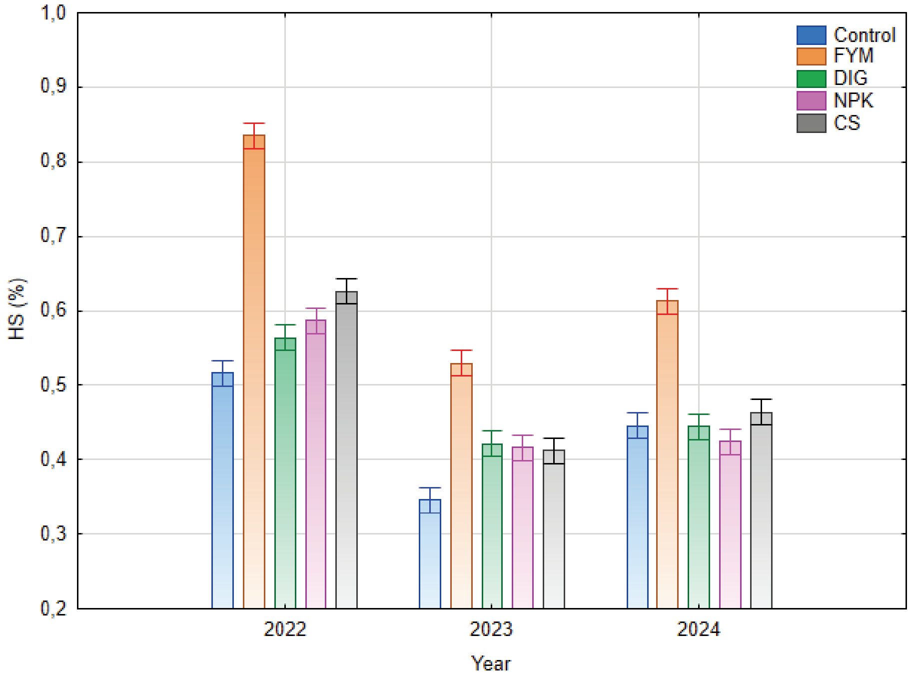



**Fig. 2.** Average humus content after amending grassland soil with organic and mineral fertilizers

Explanations: Control – control without fertilization; FYM – farmyard manure; DIG – digestate; NPK – mineral fertilization (nitrogen, phosphorus, potassium); CS – Slurry.

**Fig. 3.** Average humic substances (HS) content after amending grassland soil with organic and mineral fertilizers

Explanations: Control – control without fertilization; FYM – farmyard manure; DIG – digestate; NPK – mineral fertilization (nitrogen, phosphorus, potassium); CS – Slurry.

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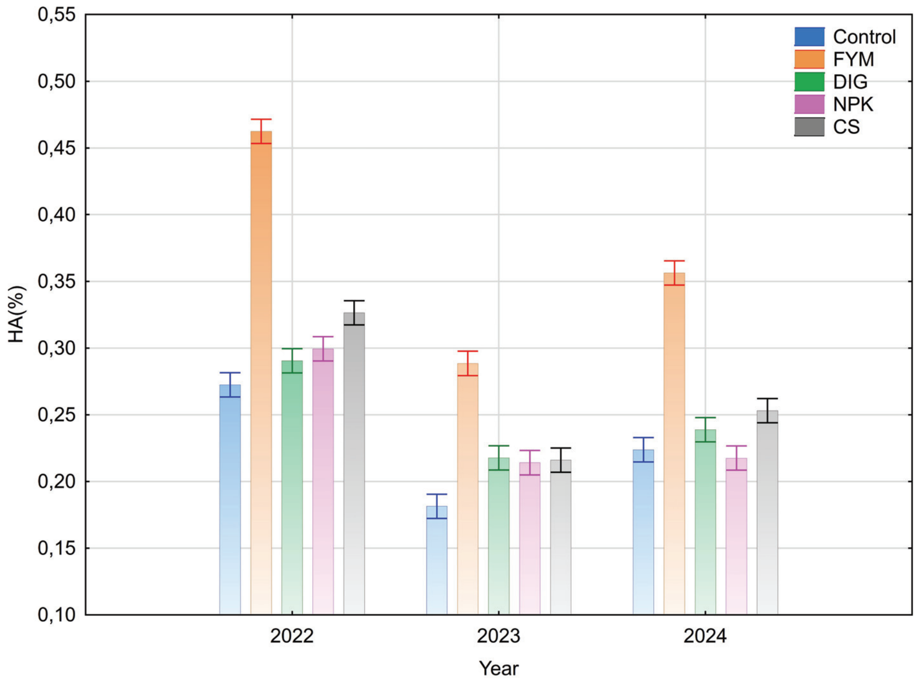
Other important parameters determined from alkali extraction and HS fractionation are humic acids (Fig. 4) and fulvic acids contents (Fig. 5). Their ratio (Fig. 6) determines the HS character and quality in studied variants. Obtained values are always higher than one, and year variability is evident from Fig. 6. In spring 2024, the highest values on FYM (1.24) were measured, which means the high quality of HS and prevalence of more stable and highly humified HA. These results are in accordance with the literature and were published by Voltr et al. (2021). The results were statistically significant when compared with the control and other variants (DIG, CS, NPK). In spring 2023, the HA/FA ratio on FYM (1.20) was significantly different compared to DIG (1.07) and NPK (1.05) variants. Evaluation according to HA/FA ratio showed high quality of HS in grassland soil (Fig. 6). Calculated humification degree (HD=HA/SOC*100) was low and varied between 10 – 20%. Differences between the studied variants were not statistically significant. It can be concluded that fertilization had a positive effect on the amount of humified stable HS, HA and FA.

The effect of fertilizing on HS quality is documented by higher absorbance in the UV-VIS spectral range (Fig. 7). It is shown that during the studied period, the HS absorbance is higher after amending the soil with all types of fertilizers. UV-VIS spectral region at about 440–465 cm$^{-1}$ is typical for less stable HS with lower molecular weight. These were confirmed by Kumada (1987) and

**Fig. 4.** Average humic acids (HA) content after amending grassland soil with organic and mineral fertilizers

Explanations: Control – control without fertilization; FYM – farmyard manure; DIG – digestate; NPK – mineral fertilization (nitrogen, phosphorus, potassium); CS – Slurry.



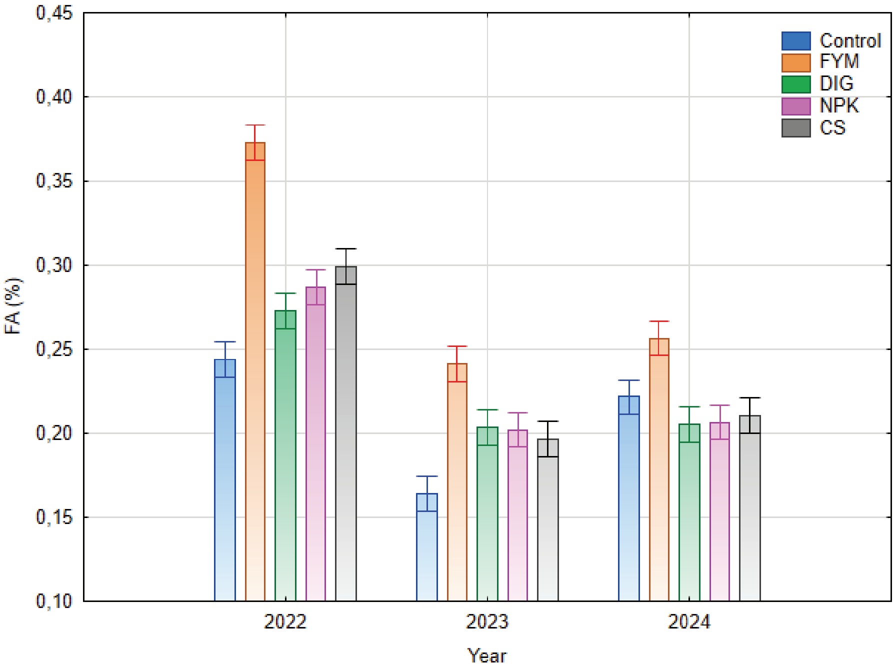



**Fig. 5.** Average fulvic acids (FA) content after amending grassland soil with organic and mineral fertilizers

Explanations: Control – control without fertilization; FYM – farmyard manure; DIG – digestate; NPK – mineral fertilization (nitrogen, phosphorus, potassium); CS – Slurry.

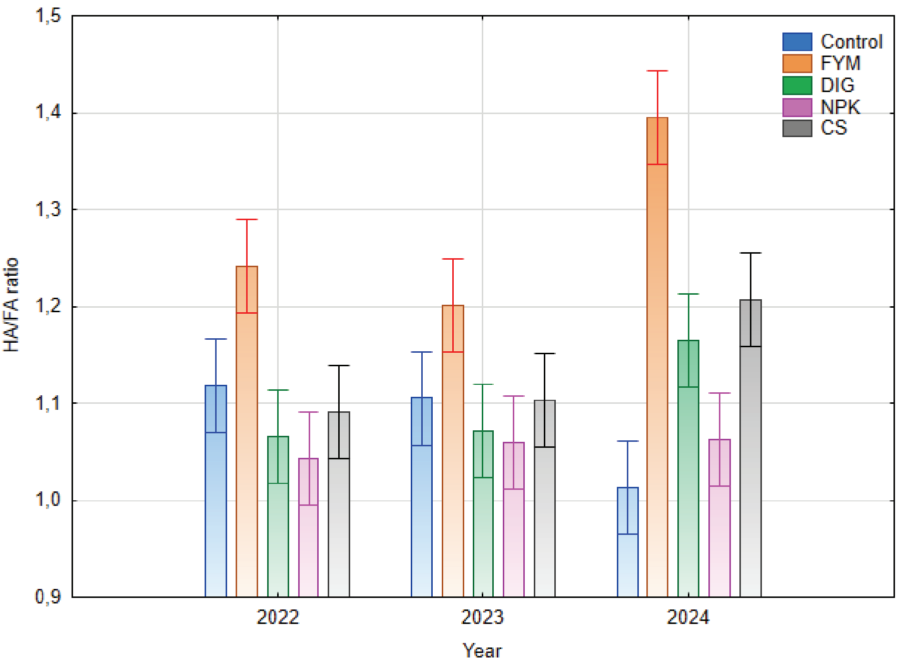


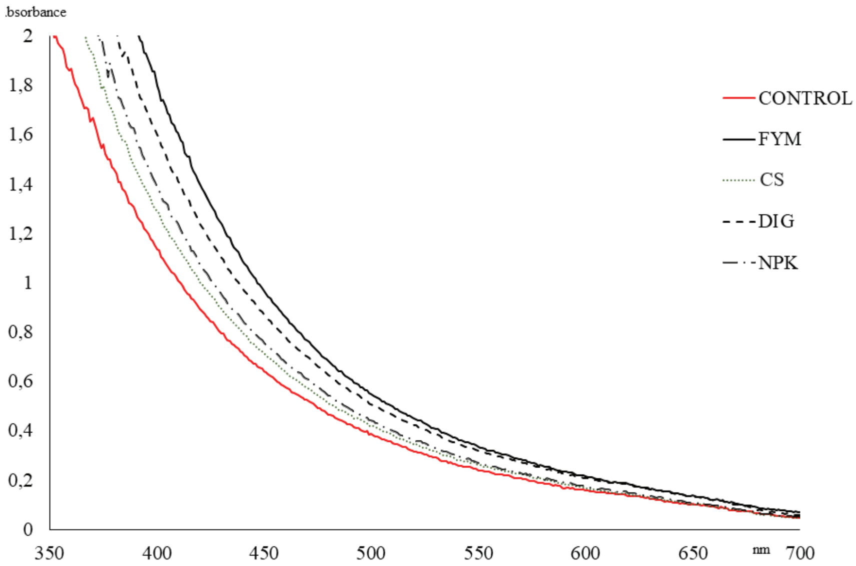

**Fig. 6.** Humic acids (HA) and Fulvic acids (FA) ratio after amending grassland soil with organic and mineral fertilizers

Explanations: Control – control without fertilization; FYM – farmyard manure; DIG – digestate; NPK – mineral fertilization (nitrogen, phosphorus, potassium); CS – Slurry.

Viscarra Rossel (2006). The region 660–665 $cm^{-1}$ is typical for HS more condensed, aromatic, stable, with higher molecular weight (Kumada, 1987, Hudec, 2012). According to the obtained results, the highest HS aromaticity and stability were achieved after FYM application. Calculated colour index (A 465/A665 nm) on FYM variant was less than 6. On the control site was colour index higher then 6. Colour index was increasing as follows: FYM < CS< DIG < NPK < Control. Similarly, Mayhew et al. (2023) and Feszterová et al. (2024) confirmed that the shape of spectral curve can suggest the composition and stability of HS. The steep spectral line progression indicates higher colour index value lower absorbance and HS stability. The steeper spectral lines are typical for Cambisols, Fluvisols and less steep progression is typical for Chernozems. This was also confirmed by our research. Comparing the results of absorbance in the UV-VIS spectral range and the classical fractional method, it was found that the HA/FA ratio was decreasing as follows: FYM < CS< DIG < NPK < Control. The obtained results confirmed that the amount and quality of HS were the highest after FYM, but all types of amending had a positive effect on SOM. DRIFT spectroscopy was applied to evaluate the chemical composition of mineral soil samples and isolated HA samples. All studied samples suggested stretching vibration and deformation of the following functional groups: carboxylic and amido- groups at 1655–1654 $cm^{-1}$; C=O bands

**Fig. 7.** Absorbance HS in UV-VIS spectral region after amending grassland soil with organic and mineral fertilizers

Explanations: Control – control without fertilization; FYM – farmyard manure; DIG – digestate; NPK – mineral fertilization (nitrogen, phosphorus, potassium); CS – Slurry.




at 1690–1716 cm$^{-1}$; carbonyl and carboxylic groups at 1719–1718 cm$^{-1}$; – C-H, $CH_2$; and $CH_3$ aliphatic groups at 2942–2920 cm$^{-1}$; amino- and amido-groups -N-H at 3500–3200 cm$^{-1}$ (Table 3). Studied samples significantly varied in the amount of labile aliphatic hydrophobic groups (marked as column A, integrated area 3000–2800 cm$^{-1}$), aromatic stable and resistant C=C groups (marked as column B, integrated area 1660–1580 cm$^{-1}$), aromatic stable groups (marked as column C, integrated 1546–1520 cm$^{-1}$), and hydrophilic amido-, carboxylic-, keto-groups (marked as column D, integrated area 1740–1600 cm$^{-1}$). Mineral soil samples showed that after amending soils with FYM, DIG, CS and NPK, there was an increase in aliphatic and labile hydrophobic groups at 2800–3000 cm$^{-1}$. The content of hydrophilic resistant groups at 1660–1580 cm$^{-1}$ was comparable with the control, except for a decrease in the FYM variant. This indicated more available organic material for soil biota after FYM application. Stable aromatic groups at 1546–1520 cm$^{-1}$ reached the highest values after FYM application, which corresponded with the formation of more stable humic acid molecules. This was also confirmed by a lower humification degree and a high hydrophobicity index on the FYM variant. On the other hand, NPK application caused a decrease in humification degree and hydrophobicity as compared to the untreated control, as well as a decrease in stable aromatic compounds at 1546–1520 cm$^{-1}$ (Table 3 and Fig. 8).

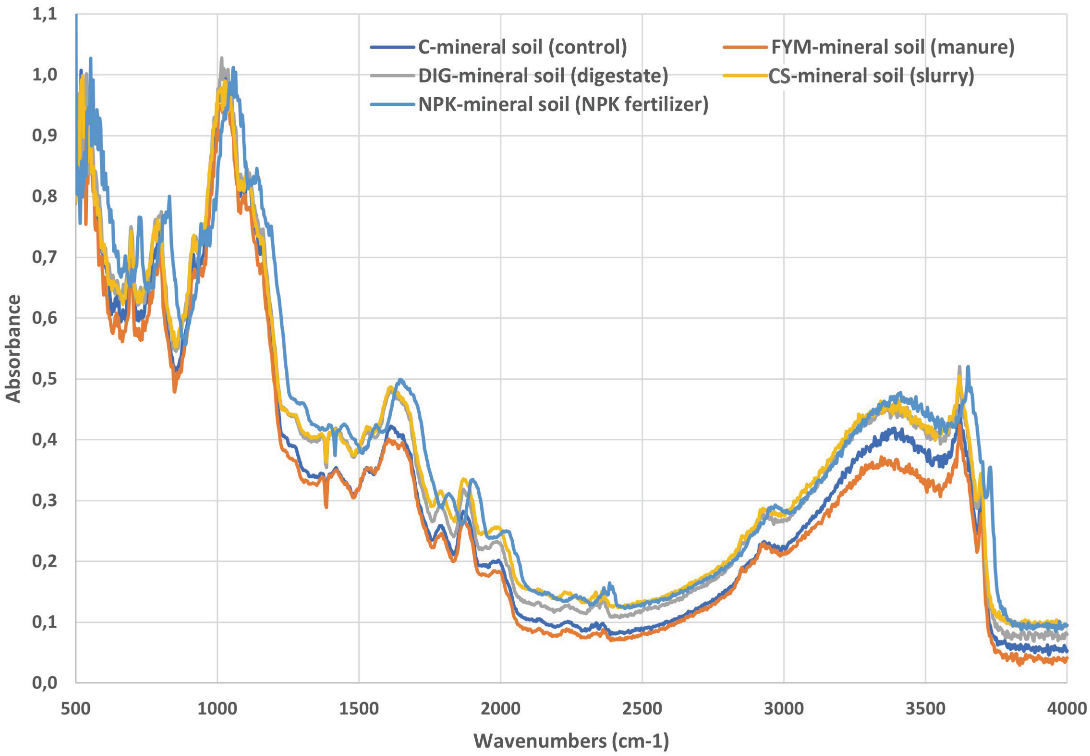



**Fig. 8.** DRIFT spectra of mineral soil samples after amending soil with mineral and organic fertilizers

Explanations: Control – control without fertilization; FYM – farmyard manure; DIG – digestate; NPK – mineral fertilization (nitrogen, phosphorus, potassium); CS – Slurry.

**Table 3**

| Variants | Intensity of DRIFT spectra | | | | Index (B+C)/A | Index A/D |
|---|---|---|---|---|---|---|
| | A | B | C | D | (Humification degree) | (Hydrophobicity index) |
| Mineral soil+Control | 2.964 a* | 1.813 b | 0.137 NS | 4.221 cd | 0.658 b | 0.702 a |
| Mineral soil+FYM | 3.522 b | 1.183 a | 0.139 | 4.077 c | 0.376 c | 0.865 e |
| Mineral soil+DIG | 3.203 ab | 1.944 b | 0.094 | 4.929 a | 0.640 b | 0.650 a |
| Mineral soil+CS | 3.330 ab | 1.864 b | 0.130 | 4.641 a | 0.600 b | 0.718 a |
| Mineral soil+NPK | 3.242 ab | 1.905 b | 0.116 | 4.919 a | 0.624 b | 0.659 a |
| HA-Control | 7.475 c | 0.457 cd | 0.129 | 6.728 b | 0.079 a | 1.113 cd |
| HA-FYM | 7.670 cd | 0.727 ac | 0.173 | 6.348 b | 0.118 a | 1.210 bd |
| HA-DIG | 7.972 d | 1.072 a | 0.190 | 6.268 b | 0.158 a | 1.272 b |
| HA-CS | 7.426 c | 0.705 ac | 0.111 | 5.705 e | 0.110 a | 1.302 b |
| HA-NPK | 5.265 e | 0.195 d | 0.228 | 5.055 a | 0.080 a | 1.043 c |

Explanations: FYM – manure + slurry, CS – cattle slurry, DIG – digestate, NPK – mineral fertilizers; *means within the column followed the same letter do not differ significantly as determined by Tukey multiple range tests ($P < 0.05$); NS – non-significant differences; column A – integrated area 3000–2800 $cm^{-1}$; column B – integrated area 1660–1580 $cm^{-1}$; column C – integrated 1546–1520 $cm^{-1}$; column D, integrated area 1740–1600 $cm^{-1}$.

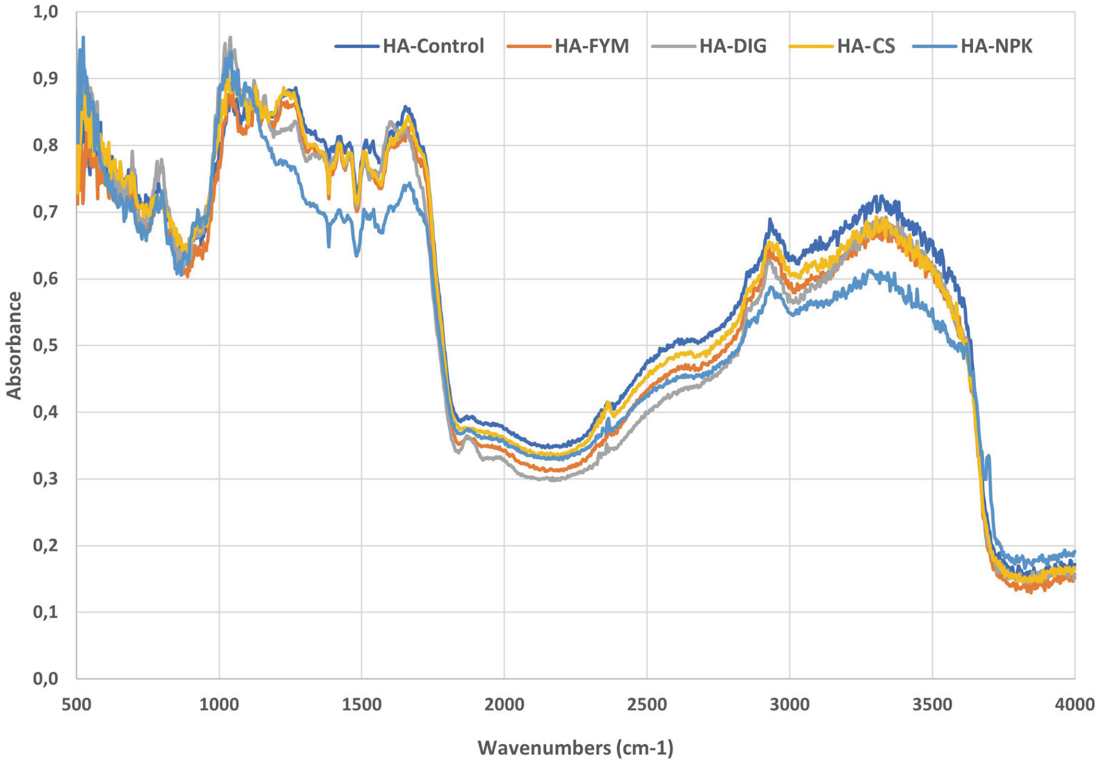



**Fig. 9.** DRIFT spectra of humic acids after amending soil with mineral and organic fertilizers

Explanations: Control – control without fertilization; FYM – farmyard manure; DIG – digestate; NPK – mineral fertilization (nitrogen, phosphorus, potassium); CS – Slurry.

Studied humic acids (HA) samples varied significantly in the amount of labile aliphatic hydrophobic groups (marked as column A, integrated area 3000–2800 $cm^{-1}$), aromatic stable and resistant C=C groups (marked as column B, integrated area 1660–1580 $cm^{-1}$), aromatic stable groups (marked as column C, integrated 1546–1520 $cm^{-1}$), and hydrophilic amido-, carboxylic-, keto-groups (marked as column D, integrated area 1740–1600 $cm^{-1}$) comparing with control untreated site (Table 3 and Fig. 9). After FYM application higher amount of labile aliphatic hydrophobic groups (columns name and integrated areas ranges were given earlier in the text) was determined. On the other hand, less hydrophilic amido-, carboxylic-, and keto-groups were determined compared with HA isolated from the control site. On the other hand, less hydrophilic amido-, carboxylic-, keto-groups (marked as column D, integrated area 1740–1600 $cm^{-1}$) compared with the HA-control site. The effect of DIG and CS application was similar and of labile aliphatic hydrophobic groups (marked as column A, integrated area 3000–2800 $cm^{-1}$), increasing of aromatic stable and resistant C=C groups (marked as column B, integrated area 1660–1580 $cm^{-1}$), and aromatic stable groups (marked as column C, integrated 1546–1520 $cm^{-1}$) was in HA molecule detected. NPK application caused decreasing of labile aliphatic hydrophobic groups (marked as column A, integrated area 3000–2800 $cm^{-1}$), aromatic stable and resistant C=C groups (marked as column B, integrated area 1660–1580 $cm^{-1}$), and hydrophilic amido-, carboxylic-, keto-groups (marked as column D, integrated area 1740–1600 $cm^{-1}$) comparing with control site (Table 3 and Fig. 9). The content of aromatic stable groups (marked as column C, integrated 1546–1520 $cm^{-1}$) increased–after NPK application. The hydrophobicity index and humification degree (index (B+C)/A) was higher after FYM, DIG, CS application and lower after NPK application. It was concluded that higher values of decomposition/humification and higher hydrophobicity on FYM, DIG and CS sites indicated enough available organic material for soil biota to compare with NPK and control sites. Coates et al. (2000) demonstrated that the amount of hydrophilic and aromatic groups and the hydrophobicity of HA can have a significant effect on the binding activity and stability of HA.

## 4. Discussion

The application of both organic and mineral treatments to grassland soil resulted in an increase in carbon stock and an improvement in the quality of SOM. However, the impact of mineral fertilization was generally lower compared to organic amendments such as farmyard manure (FYM), cattle slurry (CS), and digestate (DIG). These organic inputs contributed more effectively to SOM enrichment, likely due to their higher content of labile and bioavailable organic compounds. The NPK application caused a decrease in labile aliphatic hydrophobic groups, aromatic stable and resistant C=C groups, and hydrophilic amido-, carboxylic-, and keto-groups. Monitoring of stable soil organic matter components (HS, HA, FA) showed a statistically significant increase in the stable components (humified) after FYM application. Similar results were reported by Duong et al. (2012) and Whelan et al. (2013). They quoted that fertilization together with agrotechnical measures (e.g. intensity of grass cutting, length of grass cover) have a significant impact on the forage production and its quality. The field experiment also documented that a combination of mineral and organic fertilizers gives the most benefits for plants and the soil environment. It should also be emphasized that long-term application of mineral fertilizers (NPK) without organic amending leads to changes in SOM chemical composition, and intensive soil acidification is taking place. Egan et al. (2018) also showed that SOM status and chemical composition can be influenced by liming doses. Authors also reported that long-term mineral fertilizing led to a degradation of SOM and a decrease in SOC stocks. Nobile et al. (2020) and Xiang et al. (2020) documented that the choice of fertilizer has a great effect on SOM content and quality and may significantly improve not only nutrient status but also soil chemical properties and pH. Similarly, Deru et al. (2023) and Qi et al. (2023) showed that organic fertilizers increased SOC accumulation, which was also confirmed in our study. Rambaut et al. (2022). Xu et al. (2024) pointed out that the use of manure and organic fertilizers is not only beneficial for soil organic matter, but also these fertilizers are useful to soil biota and plant production. On the other hand, according to Bicharanloo et al. (2024) NPK application is important for grasslands because of forage biomass production, and it is also linked to the carbon cycle in the soil environment. The effect of organic fertilizers on grasslands was also studied by Karabcová et al. (2015), Bramble et al. (2024), and Lei et al. (2025), who confirmed a positive effect of organic fertilizing on forage production. Unfortunately, they did not find any increase in species diversity after organic fertilizers application. They mentioned that SOC accumulation in grassland is affected not only by fertilization but also by type of grassland and plant utilization.

## 5. Conclusions

The application of exogenous organic and mineral materials directly affected the chemical composition of soil organic matter. DRIFT spectroscopy revealed characteristic stretching and deformation vibrations of functional groups. Following amendments with FYM, DIG, CS, and NPK, statistically significant changes were observed in labile aliphatic hydrophobic groups, aromatic C=C groups, and aliphatic hydrophilic functional groups. Generally, an increase in labile aliphatic groups was determined. UV-VIS spectroscopy documented an increase in absorbance after the treatment. Compared to the classical fractionation method, spectroscopic techniques offer a faster and less labour-intensive approach for characterising soil organic matter. Further studies are needed to assess grassland productivity and forage quality after fertilization.

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### Conflict of interest

The authors declare no conflict of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. This research did not involve human or animal subjects.

### Author Contributions

**Jana Plisková** – Conceptualization, Methodology, Funding acquisition, Writing – original draft, Investigation, Sampling, Data curation, Visualization. **Lubica Pospíšilová** – Supervision for humic substances and UV-Vis spectroscopy, Writing – review and editing. **Pavel Nerušil** – Sampling, Methodology. **Tomáš Šimon** – Supervision for FTIR spectroscopy, Investigation, Data curation, Validation, Writing – review and editing. **Ladislav Menšík** – Methodology, Funding acquisition, Writing – review and editing, Supervision for grassland, Validation. All authors read and approved the final manuscript.

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