Molecular characteristics of humin fraction isolated from soils of temperate climate: a study on Chernozems and Phaeozems in Poland

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Abstract

This paper provides new information on molecular properties of the humin fraction (HM) isolated from the mollic horizons of Phaeozem and Chernozem soils developed from different parent materials in the temperate climatic zone of Poland. In our work we did not extract this fraction by dissolution but isolated it by exhaustive extraction of humic and fulvic acids with NaOH and then removing the mineral fraction with HF/HCl treatment. Obtained HM reflects the properties of this fraction that naturally occurres in the soil environment. The assessment of the structural properties of the HM was carried out using the spectroscopic methods (¹³C CP MAS NMR, FTIR, EPR), HPLC, SEM-EDX, and elemental composition analyses. The ¹³C CP MAS NMR spectra of the HM showed the advantage of aromatic structures over other organic components. The FTIR spectra confirmed that the content of aromatic structures dominated over the aliphatic ones, ranging from 7.05 to 10.32%. The EPR study indicated evidence of an unpaired electron situated on the condensed aromatic moieties. The HPLC investigation revealed the dominance of hydrophobic fractions, ranging from 77.41 to 80.83%. The ash content, ranging from 22.89 to 54.50%, as well as SEM-EDS study indicate that the isolated HM is not a pure organic fraction but constituted strongly bound organo-mineral compounds, resistant to further treatment with an alkali and HF/HCl mixture. This fraction represents the most resistant pool of the SOM that plays a crucial role in soil carbon sequestration and due to high functional group content contributes to the maintenance of soil and its ecosystem services.

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Highlights

- Humin is the hydrophobic SOM fraction playing a key role in carbon sequestration
- Humin isolated by extraction of humic acids is strongly bound to mineral fraction
- The mineral bound humin has a structure dominated by aromatic components

Abstract

This paper provides new information on molecular properties of the humin fraction (HM) isolated from the mollic horizons of Phaeozem and Chernozem soils developed from different parent materials in the temperate climatic zone of Poland. In our work we did not extract this fraction by dissolution but isolated it by exhaustive extraction of humic and fulvic acids with NaOH and then removing the mineral fraction with HF/HCl treatment. Obtained HM reflects the properties of this fraction that naturally occurres in the soil environment. The assessment of the structural properties of the HM was carried out using the spectroscopic methods (¹³C CP MAS NMR, FTIR, EPR), HPLC, SEM-EDX, and elemental composition analyses. The¹³C CP MAS NMR spectra of the HM showed the advantage of aromatic structures over other organic components. The FTIR spectra confirmed that the content of aromatic structures dominated over the aliphatic ones, ranging from 7.05 to 10.32%. The EPR study indicated evidence of an unpaired electron situated on the condensed aromatic moieties. The HPLC investigation revealed the dominance of hydrophobic fractions, ranging from 77.41 to 80.83%. The ash content, ranging from 22.89 to 54.50%, as well as SEM-EDS study indicate that the isolated HM is not a pure organic fraction but constituted strongly bound organo-mineral compounds, resistant to further treatment with an alkali and HF/HCl mixture. This fraction represents the most resistant pool of the SOM that plays a crucial role in soil carbon sequestration and due to high functional group content contributes to the maintenance of soil and its ecosystem services.

Keywords: soil organic matter composition; humin molecular structure; soil organo-mineral complexes; humic substances; spectroscopic methods

1. Introduction

Soil organic matter (SOM) constitutes one of the key indicators of soil quality, due to its significant role in many interconnected chemical and biochemical processes occurring in the soil. The transformation of SOM, related to its decomposition and humification, depends on many environmental factors that may modify the ongoing changes. Soils that are considered to be the largest carbon pool contain more organic carbon than is contained in the atmosphere and vegetation together (Minasny et al 2013). Therefore, the stabilization of SOM may greatly affect carbon capture and immobilization to efficiently limit global climate change (Bhattacharyya et al., 2022; Liu et al., 2022; Pham and Katayama, 2018; Spaccini et al., 2002). Due to the complicated matrix and heterogeneity of SOM, the recognition of the chemical diversity of its individual components is significant in the processes of controlling its transformations (Kibblewhite et al. 2008, Rabot et al. 2018, Wiesmeier et al. 2019).

Decades of research have shown that to recognize SOM's role in the environment, it is necessary to separate it into components with different properties (Hayes et al. 2017, 2020). Although more than 230 years have passed since the first extraction of humic substances (HS) by Achard, the nature, structure and fractionation of organic matter are still the subject of scientific discussion, and today there is no consensus on which approach should be applied (Gerke, 2018; Janzen, 2019; Olk et al., 2019a and 2019b; Schnitzer and Monreal, 2011; Weber et al., 2018). Recently, several new concepts concerning SOM isolation and fractionation have appeared, including physical separation methods (Cambardella and Elliott, 1992; Lavallee et al., 2020) that base on size and density. The last decade has brought more and more heated debate after Lehmann and Kleber (2015) called into question several established views and concepts regarding SOM, including the occurring and the formation of HS. This concept is controversial and does not have the approval of many SOM researchers (De Nobili, 2019; Hayes and Swift, 2020; Olk et al., 2019a, 2019b). Obviously, humic fractionation, like any procedure, has limitations; however, any procedure can and does provide useful results (Olk et al., 2019a; Zaccone et al., 2018).

According to the widely accepted proposal of Hayes and Swift (1978), which is in line with the classical approach, "SOM is a heterogeneous mixture of all organic components found in soil, which can be subdivided into" (1) unaltered materials and (2) transformed products which consist of both: (2a) HS and (2b) non-humic substances. According to the International Humic Substances Society, HS "are complex and heterogeneous mixtures of polydispersed materials formed in soils, sediments, and natural waters by biochemical and chemical reactions during the decay and transformation of plant and microbial remains" (https://humic-substances.org/what-are-humic-substances-2/). Some researchers (Piccolo, 2002; Sutton and Sposito, 2005) are inclined to the view that "humic substances are collections of diverse, relatively low molecular mass components forming dynamic associations stabilized by hydrophobic interactions and hydrogen bonds". HS are divided into three main groups: humic acids (HA), fulvic acids (FA), and humin fraction (HM). The HA and FA can be extracted from the soil with a strong alkali, then the HA is separated by adding a strong acid, which causes their precipitation. The HM can HM can be extracted with neither a strong base nor a strong acid (Aiken et al., 1985; Hayes et al., 2017; Rice and MacCarthy, 1988), which is why some researchers do not include this fraction in HS, while others do. Thus, HM research could be the "bridge" between more traditional and newer theories of SOM investigation.

The HM usually constitutes more than 50% of HS, but the resistant chemical nature of this fraction as well as its lack of solubility contribute to a significant difficulty in its investigating. Thus, although it is a main component of SOM, the composition and properties of the HM have rarely been studied. Since its structure is close to that of HA, but slightly more aliphatic (Hatcher et al., 1985; Tan, 2014), some authors claim that the HM is formed as a mixture of highly condensed HA bonded to fine mineral soil particles (clay minerals), melanin of fungal origin, paraffinic compounds (Stevenson, 1994), lignin from plants or peptidoglycan structures from microbial cells (Simpson et al., 2007). Hayes et al. (2017) indicated that the backbone of humin is predominantly composed of "aliphatic hydrocarbon functionalities, especially those found in lipids, waxes, cuticular materials, cutin, cutan, suberin, and suberin". However, the HM is strongly bonded physically and chemically to HA and FA fractions associated with "a disordered matrix entrapping a significant proportion of non-polar compounds" (Almendros and González-Vila, 1987; Song et al., 2011). Generally, the HM revealed a particle diameter smaller than the FA and HA, and according Ukalska-Jaruga et al. (2021) "a more spherical shape caused by the higher intermolecular forcing between the particles". In addition, the aromatic-aliphatic carbon core structure of the HM also depends on its origin and soil depth (Tatzber et al., 2007). Simpson et al. (2007) showed that all HM species "are present as macromolecules (or stable aggregate species)" and belong to "five main categories of components, namely, peptides, aliphatic species, carbohydrates, peptidoglycan and lignin". Song et al. (2011) isolated and fractionated the HM with alkaline urea and acidified dimethyl sulfoxide (DMSO), suggesting that the recalcitrant HM consists of molecules originating "from plants and the microbial population, that are likely to be protected from degradation by their hydrophobic moieties and by sorption to the soil clays".

Despite the difficulties in HM studies due to their insolubility, there has been growing attention to the issues related to its genesis and role in soil and carbon sequestration (Pham et al., 2021; Schnitzer and Monreal, 2011, Tang et al., 2022; Zhang and Katayama, 2012). To assess this, it is necessary to obtain as much data as possible on the properties of the HM of soils derived from different parent materials under diverse vegetation and climatic conditions. HM is most often extracted from soil material by organic solvents, whereas in our work we did not extract this fraction by dissolution but isolated it by eliminating other organic and mineral components from the soil material. As a consequence, investigated humin as a strong organo-mineral complexes reflects the properties of this fraction that naturally occurres in the soil environment. The main aim of the research was to assess the molecular properties of the HM obtained from various arable soils in the temperate climate zone, using spectroscopic methods (¹³C CP MAS NMR, FTIR, and EPR), HPLC, SEM-EDX, and elemental composition analyses.

2. Materials and methods

2.1. Soil location and characteristics

Soil samples were collected from the upper 30 cm of eight mollic horizons of Chernozems and Phaeozems developed from different parent rocks. They were used as arable soils with different plants grown in various regions of Poland (Table 1). The HM samples were labelled 11K, 10PY, 9H, 8C, 7T, 6M, 3Z, and 1PS, which refer to the sampling location by the number and first letter(s) of the names of the nearest towns or villages.

Investigated soils were developed under diverse agroecological conditions and exhibited different basic properties (Table 2). They indicated different pH (5.64 - 7.71), TOC contents ($13.3 - 41.7 \text{ g kg}^{-1}$), and CEC values ($21.6 - 53.2 \text{ cmol}(+) \text{ kg}^{-1}$) as well as textures (from sandy loam to clay), thus representing various soils cultivated in temperate climatic conditions.

2.2. Isolation of humin

The detailed isolation procedure of the HM was described in our previous work (Weber et al., 2022). Briefly, the applied method was based on the classic separation of the HS by eliminating the alkali-soluble SOM fractions (HA and FA) from the soil matrix by exhaustive 0.1 M NaOH extraction. Next, the remaining mineral fraction was digested with a 10% HF-HCL mixture. The collected HM was freeze-dried and then subjected to spectral studies to assess its molecular characteristics. All analyses were performed on solid HM samples (excluding HPLC analyses).

2.3. Elemental composition

The elemental composition of the HM was analyzed with the CHNS Vario EL Cube analyzer (Elementar; Langenselbold, Germany) in three repetitions to obtain the highest precision of results. The contents of C, H, N, and S were measured as percentages of the ash-free mass, while the oxygen concentration was calculated from the difference. The atomic ratios H/C, O/C, O/H, and N/C were calculated to show the relationship of the elements in the composition of the HM. Furthermore, the degree of internal oxidation (ω) was determined from the atomic percentage contents (Tan, 2014), according to the formula: $\omega = [(2O + 3N) - H]/C$.

2.4. 13 C CP MAS NMR

The ¹³C CP MAS NMR spectra were acquired with a Bruker Avance III spectrometer (Bruker Inc., Germany) at 300 MHz, equipped with a 4-mm MAS probe and operating in a ¹³C resonance sequence at 75.48 MHz. The HM samples were placed in a rotor (sample holder) of zirconium dioxide (ZrO_2) with Kel-F caps, with a rotation frequency of 10 kHz. The spectra were obtained by collecting 4994 data points from the same number of scans at an acquisition time of 50 ms and with a 4 s recycle delay. Spectral collection and elaboration were performed using Bruker Topspin 3.6 software. The areas of characteristic functional groups were calculated by integration of appropriate parts of the spectra using Bruker Topspin 4.1.1. software. Hydrophobicity (HB) was calculated as follows:

HB = [(0-57 ppm) + (116-151 ppm)] / [(57-116 ppm) + (151-220 ppm)] (Xu et al., 2017).

2.5. FTIR

The FTIR spectra were measured using a Bruker Vertex 70 FTIR spectrometer with KBr pellets (approximately 1 mg of sample in 400 mg of KBr). Collected spectra were presented as transmittance. For the chosen peaks: -C, -O, and -OH deformations of COOH (1190–1300 cm⁻¹), aromatic -C=C- (1570–1677 cm⁻¹), -C=O of COOH (1677–1800 cm⁻¹), $-CH_2-$ symmetric stretch (2796–2850 cm⁻¹), and $-CH_2-$ asymmetric stretch (2877–2985 cm⁻¹) integrations were performed (Machado et al., 2020; Swift, 1996; Tatzber et al., 2007). The integral areas were calculated from absorbance spectra using OriginPro 2016 and OriginPro 9.5 software.

2.6. EPR

The X-band EPR spectra were obtained with a Bruker Elexsys E500 spectrometer at room temperature with the use of the double rectangular cavity resonator devoted to quantitative measurements. More details could be found in the work of Pospíšilová et al. (2020). The HA standard of Pahokee peat (1S103H) and the Leonardite standard HA (1S104H) distributed by the International Humic Substances Society, in addition to the Bruker alanine pill, were used as quantitative standards. To quantitatively analyze the spectra of the HM radicals, double integration of the signals was performed using WinEPR by Bruker.

2.7. HPLC

The hydrophilic-hydrophobic properties of the HM were determined using a Perkin-Elmer HPLC Series 200 liquid chromatograph equipped with a fluorescence detector. Chromatographic separation was carried out on an analytical column X-Terra C18 with a particle size of 5 μ m and dimensions of 250 × 4.6 mm ID. The HM samples (5 mg) were dissolved in 2 cm³ of DMSO-H₂SO₄mixture (94:6 v/v) and filtered through a 0.45- μ m PVDF syringe filter. For the chromatographic separation, an eluent of acetonitrile-water and a gradient elution program were used. The injection was 10 μ L, and the detection was at excitation/emission wavelength (λ ex/ λ em) 270/330 nm. Based on the determined areas under the peaks, the share of hydrophilic (HI) and hydrophobic fractions (HB) as well as the HI/HB parameter were calculated.

2.8. SEM EDS

Scanning electron microscopy (SEM) analysis of the HM ash was performed with a Hitachi S-3400N SEM, equipped with a tungsten cathode attached with energy-dispersive X-ray spectroscopy. Measurements were made at a pressure of 30 Pa and an accelerating voltage of 30.0 kV. The images were captured using backscattered electrons to better visualize the material contrast. The EDS analyses were performed with a Noran System7 analyzer with a Thermo Scientific Ultra Dry detector, with a resolution of 129 eV.

2.9. Statistical analysis

OriginPro 2016 and OriginPro 9.5 software were used for the elemental analysis and the concentration of EPR radicals. The software package Statistica (Dell Statistica, version 13.1) was used for the principal component analysis (PCA) and cluster analysis. These analyses were used to investigate the relationships among the determined humin molecular characteristics. In order to identify the main correlation structure of the measured variables, their inter-relationships and hidden structures were evaluated with PCA, whereas cluster analysis was performed to identify dependent groups.

3. Results and discussion

3.1. Elemental composition

The soils analyzed showed varied HM contents ranging from 37.09 to 65.05% of TOC (Table 2), which confirms the great diversity of the soils analyzed. These data are in good agreement with results obtained by other authors (Grasset and Ambles, 1998; Zhang et al., 2019), highlighting the large share of the HM in SOM. The elemental composition of the HM is presented in Table 3. The ash content in the HM ranged from 22.89 to 54.50%, which is within the typical range for mineral soils (Stevenson, 1994; Tan, 2014) and was probably due to the different strengths of organo-mineral bonds, nevertheless confirming a strong association of the HM with mineral colloids probably related to the processes of their stabilization in the soil (Hayes et al., 2017; Pham et al., 2021; Simpson and Johnson, 2006; Tan, 2014; Zhang et al., 2015). The ash content was not correlated with clay fraction (< 0.002 mm) nor with TOC, indicating that its share depends mainly on the strength of organo-mineral bonds, protecting the mineral fractions from destruction during the treatment of the sample with HF/HCl (Weber et al., 2022).

The content of elements varied in the range of 42.02–58.86% for C, 3.88–4.77% for H, and 2.52–3.68% for N, and this affected the atomic ratios given in Table 4. It is generally assumed that the increase in humification is associated with a decrease in C/N, H/C, and O/C ratios in response to a relative enrichment with total N relative to organic C, as well as a residual enrichment of more recalcitrant aromatic and aliphatic compounds (Zaccone et al., 2018). The low H/C ratio indicated the dominance of aromatic structures (Rice and MacCarthy, 1988; Zhang et al., 2015) in the investigated HM samples.

The degree of internal oxidation (ω) is another parameter that clearly indicates the quantitative predominance of hydrocarbon structures over functional groups containing oxygen and nitrogen (Table 4). This parameter is considered significant for the analysis of diagnostic changes in HS that are related to degradation and oxidation reactions (Tan, 2014). The HM of the analyzed soils showed different values of this parameter, ranging from 0.020 to 0.672. These differences may indicate diversity in aerobic conditions related to soil pedogenesis. The HM with the lowest degree of oxidation was isolated from the Gleyic Phaeozem (8C) and developed under weak drainage conditions. Haplic Chernozem (7T) is a well-drained soil with much better aerobic conditions, which favors the oxidation of humic substances (Cieslewicz et al., 2008; Tan, 2014), resulting in the highest (0.672) ω value. However, not all Gleyic Phaeozems (6M, 10PY, and 11K) exhibited a comparatively low degree of internal oxidation.

3.2. ¹³C CP MAS NMR studies

The NMR spectra obtained (Figure 1) demonstrated the classical pattern observed for the HS (Hayes et al., 2017; Swift, 1996). All recorded spectra were very similar to each other and indicated several functional groups typical for organic substances. The alkyl region (18–57 ppm) includes peaks assigned to saturated hydrocarbons, from primary to quaternary, including alkanes and most of the alkyl groups. In the case of the HS, these peaks can be attributed to methylene C in organic acids (Almendros et al., 2018). In this region, a sharp peak typical for the methyl –CH₃groups was detected in all observed spectra. The region between 57 and 73 ppm is assigned to methoxyl carbon, but also to N-substituted alkyl C, such as the C α of amino acids (Almendros et al., 2016). In the presented HM in this ppm range, one weaker but broad peak at 65 ppm was detected from alkylamine C and O-alkyl C derived primarily from lignin (Swift, 1996). A more intense peak at 82 ppm was typical for carbohydrates (Preston, 1996; Preston et al., 1994; Schnitzer et al., 2006). Peaks in the region between 102 and 116 ppm can be assigned to anomeric C, sp3 carbon bonded to two O atoms (Swift, 1996). In all HM studied, the peak at about 104 ppm was relatively weak (carbohydrate shift) and exists more as a hump on the much more intense peak of aromatic C (116–151 ppm). At the highest chemical shift (~185 ppm) in all the studied samples spectra, the C peak from the carboxylic group was found.

The NMR spectra of the HM samples were integrated with the areas characteristic of particular functional groups. This indicates the predominance of aromatic structures over other organic components (Table 5), according to the results of the elemental composition, in which the H/C ratio confirms the high aromaticity of the investigated fraction.

The discussed spectra differ from those obtained by Song et al. (2011), who used an alkaline solvent enriched with urea (0.1 M NaOH + 6 M urea) and acidified DMSO solvent (DMSO: H_2SO_4 ; 94:6 v/v). These HM samples were devoid of organo-mineral bounds and showed a predominance of aliphatic structures with only a negligible presence of aromatic compounds. However, as studied by these authors, the fraction of the HM insoluble in DMSO obtained from Terra Preta soil exhibited higher amounts of aromatic structures and was more similar to the HM described in this work. The findings of Song et al. (2011) suggest that the HM obtained from various soils may differ in properties, and this confirms the need to analyze this fraction obtained from many soils developed under different environmental conditions. On the other hand, the dominance of aromatic structures in the HM studied indicates the presence of bound-HA strongly associated with the mineral fraction that protected them during NaOH extraction (Weber et al., 2022). This suggests the mutual protective effect of organic and mineral colloids (clay minerals, metal oxides, and hydroxides) present in organo-mineral bounds. Moreover, these observations confirm the processes of organic matter transformation and the interpenetration of the HA to the stable HM as a result of complexation processes (Yang et al., 2017). These components could be removed by alternating treatments with NaOH and HF/HCl to give a pure organic HM completely free of alkali-soluble components. Nevertheless, the HM samples obtained in this way would not have represented the HM naturally occurring in the soil environment, where they are strongly bound to the mineral fraction. These strong organo-mineral bonds of the HM represent the most resistant fraction of the SOM, which plays a key role in carbon sequestration in the soil environment.

3.3. FTIR spectroscopy

The FTIR spectra of the investigated HM (Figure 2) represented a similar pattern, typical for SOM (Helal et al., 2011; Mastrolonardo et al., 2013; Stevenson, 1994; Swift 1996; Tatzber et al., 2007; Ukalska-Jaruga et al., 2023). They are good indicators of qualitative characteristics, with typical bands distinguishing the humic materials. They consisted of the same peaks, with some differences observed in the region below the wavenumber of 700 cm⁻¹. This can be attributed to various compositions of inorganic constituents of the studied HS (Chen et al., 2015).

Although the organic parts of the spectra followed a similar pattern, it was possible to find differences in their intensities. Based on the transmittance, five peaks were chosen for quantitative analysis of their absorbance spectra integrated areas: 1190–1300 cm⁻¹ (–C, –O, and –OH deformations of COOH), 1570–1677 cm⁻¹ (–C=C– stretching of aromatics), 1677–1800 cm⁻¹ (–C=O stretching of COOH), 2796–2850 cm⁻¹ (–CH₂– symmetric stretching), 2877–2985 cm⁻¹ (–CH₂– asymmetric stretching) (Machado et al., 2020; Swift, 1996; Tatzber et al., 2007). Among all the HM examined, the contents of aromatic structures dominated over aliphatic ones, ranging from 7.05 to 10.32% (Table 6). The 9H sample was characterized by the largest area under the spectral curve associated with aromatic structures. The investigated HM exhibited different shares of particular areas, but the differences were too scarce to show their dependence on the properties of soil material and/or soil genesis.

Studies of 13 C-labeled HA (Spaccini et al., 2002) showed that, during incubation, the methyl group was oxidized to a carboxyl group, indicating that despite its hydrophilicity, the resulting carboxylic carbon was sequestered in the hydrophobic domain in soil. These results suggest that microbial mineralization of unstable organic compounds can be effectively reduced in soil by humified SOM, which in turn can increase the biological stability of SOM soil, and thus contribute to a significant reduction of CO₂emissions from agricultural soils.

3.4. EPR measurement

The ¹³C NMR and FTIR analyses showed that the highest content of aromatic groups was in the 9H sample. The same sample was also characterized by the highest concentration of radicals (Table 7) measured by EPR spectroscopy, which confirms that radicals in the HM are known to have an aromatic structure (semiquinones, structures with an aromatic ring substituted with O).

The similar and low values of the g-parameter ranged from 2.0029 to 2.0031, pointing out the very similar structure of the radicals in all the investigated HM and their predominantly aromatic structure. The values of the g-parameter of the investigated HM were smaller than those usually observed for the HA and FA (Jerzykiewicz et al., 1999; Jezierski et al., 2000), which is evidence of an unpaired electron situated on the more aromatic moieties (Gerson and Huber, 2003). Small differences in the structures (reflected by small g-parameter change) depend on the stability of the aromatic core of the semiquinone matrix (Jerzykiewicz et al., 1999; Jezierski et al., 2000; Gerson and Huber, 2003). It can therefore be assumed that the reactivity of the HM will be similar and dependent on the functional groups located at the aromatic rings. This indicates that the differences in the HM structures occur mainly in the forms of the aromatic core stabilizing the structure of this fraction, which is only to a small extent capable of chemical interactions.

3.5. HPLC analyses

The HPLC analysis of the HM resulted in its separation into hydrophilic and hydrophobic fractions (Table 8). The amounts of hydrophilic fractions in all investigated HM were low; thus, they indicated a significant share of hydrophobic fractions, ranging from 77.41 to 80.83%. The high share of hydrophobic fractions resulted in low values of the HI/HB parameter at the level of 0.237–0.292. Since the HM has not been a frequent subject of HPLC studies on the hydrophobicity index, it is difficult to compare our results with those known in the literature. This revealed that the HI/HB ratios for HM are lower than those known for the HA (Debska et al., 2010), indicating a higher content of hydrophobic components in the HM than in other HS (Rusanov and Anilova, 2009). On the other hand, the observed hydrophobicity coefficients are consistent with the data obtained from ¹³C NMR. Nevertheless, the aromatic compounds probably derived from the bound-HA fraction are in the form of HA occluded in HM structures – following the theory of interfacial SOM phases (Schauman 2006a, 2006b; Pignatello 2012).

Hydrophobicity can also be calculated from NMR spectra (Xu et al., 2017) or FTIR spectra (Matejkova and Simon, 2012). The observed hydrophobicity coefficients obtained from chromatography are consistent with the data obtained from 13 C NMR. However, calculations of hydrophobicity based on the FTIR spectra do not appear to be correct (data not shown). In this method, only two intensities (at approximately 1600 and 3000 cm⁻¹) are considered. In the obtained spectra, bounds at 1600 cm⁻¹ (considered hydrophilic) are always more intense than those at 3000 cm⁻¹, suggesting the dominance of hydrophilic groups over hydrophobic ones. This contradicts our other results and general knowledge about HM properties. The problem is most likely related to the limited number of regions chosen to determine the hydrophobicity index. This approach assumes that only aliphatic groups (3000 cm⁻¹) are included in calculations, whilst aromatic ones are ignored, which especially in the case of HM may result in a great falsification of the results. In addition, aromatic group bands may occur in several places and could overlap slightly with the band around 1622 cm⁻¹ (Machado et al., 2020; Swift, 1996; Tatzber et al., 2007), which is considered exclusively hydrophilic in this method (carboxylic group vibrations). Hydrophobicity calculated from 13 C CP MAS NMR spectra for different carbons gives results much closer to those obtained for HPLC, although it is difficult to correlate them.

The lowest HI/HB ratio for the 9H sample supports the results discussed above, distinguishing this particular HM sample as having the most aromatic and condensed structure (Tables 5 and 6). In general, mutual proportions of the hydrophilic and hydrophobic fractions in the HM indicate the direction of SOM transformations, which may affect the balance of organic carbon in the environment (de Aguiar et al., 2022) and contribute to its sequestration (Song et al., 2018). Spaccini et al. (2002) indicated that the higher the hydrophobicity of the humic material, the larger the sequestration of organic carbon in the soil, thus contributing to a significant reduction in CO_2 emissions. Furthermore, a large share of hydrophobic fraction in the investigated compounds indicated a sufficient content of substances to form stable soil aggregates, which was noted by Capriel et al. (1990), who obtained a positive correlation between the hydrophobic components and the stability of the aggregates.

3.6. SEM-EDS study

To characterize the mineral fraction bound to the HM more precisely, ash samples were analyzed with SEM-EDS. Analyses revealed that the ash consisted mainly of Ti (10–25 weight%), Al (4–22 weight%), Mg (3–14 weight%), and Zr (0.05–14 weight%), depending on the sample area studied (Figure 3). This indicates the dominance of oxide forms with a small share of silicates in the mineral part of the investigated HM.

To obtain more information on the mineralogical composition of the inorganic components of the HM, isolated fraction was analyzed by X-ray diffraction. The results of the XRD analysis (data intended for a separate publication, not presented in the work) confirmed that the mineralogical composition of the HM organo-mineral complex consisted mainly of anatase (TiO_2), rutile (TiO_2) and a smaller amount of sillimanite (Al₂SiO₅). These minerals are known to be stable, the most resistant to weathering processes.

3.7. Mutual relations between determined molecular characteristics of HM

Due to the numerous parameters obtained during HM analyses, cluster analysis and principal component analysis were performed to assess their mutual relationships (Figure 4). The determined HM properties were concentrated in two groups of interrelated factors: group 1 covered qualitative factors and was related to the molecular properties of the HM; group 2 covered quantitative factors, which are dependent on the fractional composition and shares of individual fractions in the total content of organic carbon. According to the statistical data (Table 9), the parameters of one group are not dependent on the parameters of the second group, i.e. the SOM fractional composition does not affect the molecular properties of the HM. Therefore, it can be assumed that the molecular properties of the HM depend on the factors affecting the quality of soil organic matter, like humin precursors and/or the soil's biological activity. These are individual features of each microspace where humification processes take place.

In the PCA analysis, the first three factors were significant and dominated the overall assessment of the mutual influence of the investigated HM properties, which was assessed on the base of the scree curve. Generally, the first three principal components accounted for 84% of the total variance of the results, while up to 66% of the variance was connected with the first two factors (Figure 5 and Table 9). The first PCA component (PCA 1) that explained 37% of the variance, was significantly correlated with elemental composition and their proportions in the HM structures (r = 0.79, r = 0.68, and r = 0.74 for H/C, O/C, and N/C, respectively), aromaticity, and related functional groups. such as COOH, O-C-O, and CH-OH (r = -0.80, r = 0.73, r = 0.88, and r = 0.61, respectively) as well as the internal oxidation parameter (r = -0.80, r = 0.73, r = 0.88, and r = 0.61, respectively)(0.59) and the g parameter (r = -0.82). The second PCA component (PCA 2), representing merely 29% of data variance, indicated significant correlation with radical concentration (r = 0.73), as well as parameters of hydrophobicity and hydrophilicity (r = -0.75, r = 0.75, and r = 0.74, for HI, HB, and HI/HB proportion, respectively). Moreover, this component was also related to hydrophobic functional groups and ω . The third component (PCA 3) accounted for 18% of the variation and was related to reactive carbon functional groups such as CH–OH (r = 0.68), OCH₃ (0.69), CH₃ (r = 0.82), and COOH (r = 0.68). The observed correlations indicate that the individual HM properties are interdependent and determine the total chemodiversity of this fraction.

Moreover, the obtained results allowed to identify homogeneous groups (Figure 6) with similar properties, i.e., 8C, 3Z, and 1PS (first group), 10PY and 11K (second group), 6M and 7T (third group), and 9H (fourth group). These identified HM groups with similar molecular properties were isolated from soils with different

characteristics, but no correlation was found between the individual specific properties of these soils. This confirms that the individual properties of HM may depend more on humification processes than on soil characteristics. Nevertheless, the number of soils to be analyzed should be extended to reliably assess the distinctive features of the HM.

4. Conclusions

The studied HM that originated from mollic horizons of different Chernozems and Phaeozems derived in temperate climatic conditions of Poland indicated a condensed structure with a predominance of aromatic components. The predominance of aromatic structures reflects hydrophobic moieties of the HM, which usually positively affect the stability of soil aggregates.

The presented results showed a high similarity of the studied HM, suggesting that the properties of the soil parent material slightly affected their structures. However, the HM, which shows the greatest resistance to decay and plays a key role in carbon sequestration, requires further research in soils derived under differentiated conditions.

The HM isolated with the adopted method was not a pure organic fraction but consisted of strongly bound organo-mineral compounds, resistant to further treatment with an alkali and HF/HCl mixture. The reason for this is that the remaining organo-mineral bonds are too strong to be destroyed by exhaustive extraction of HA and FA. Because the organo-mineral bonds also protect the mineral fraction from destruction, the separation of the pure organic humin fraction portion would require the destruction of the organo-mineral bonds by alternating NaOH and HF/HCl treatment. The HM samples obtained in this way would be useful for basic research to better understand the structure of the obtained mineral-free HM, but it seems that investigation of HM existing as strong organo-mineral complexes characterized in this work better reflects the properties of this fraction, naturally occurring in the soil environment. It is necessary to answer the question of whether the HM is insoluble in alkaline solutions during the exhaustive extraction of the organo-mineral bonds present in the soil. The isolation procedure used in this work led to the separation of the HM, which represents a highly resistant SOM fraction strongly bound to minerals, and the properties well reflect the role of this fraction in the soil system.

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