Supplementary Materials for the manuscript submitted to Agronomy:

**Polarity-based sequential extraction as a simple tool to reveal the structural complexity of humic acids**

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**Supplementary section 1:**

**Isolation and characterization of source humic acids**

**Humic acid isolation procedure**

Peat-bog humic acid (SBPHA) was extracted from South Bohemian peat using a modified procedure recommended by the International Humic Substances Society (Hanc et al., 2019; Swift, 1996). Briefly, air-dried sample was demineralized with 0.1 M HCl at a ratio of 1:10 (sample/solution), and the suspension was then washed with deionized water until an almost neutral pH was achieved. Sample was shaken overnight with 0.1 M NaOH under N₂ using sample/extractant ratio of 1:10. After centrifugation at 2700 × g for 45 min, peat HA was precipitated from the supernatant by means of acidification to pH < 2 with 6 M HCl, and further separated by centrifugation at 2700 × g for 30 min. Precipitated peat HA was treated (twice) with a mixture of 0.5 vol% HCl-HF over a period of 48 h, and subsequently separated in the centrifuge. Peat HA was purified and dialysed using a Spectra/Pos cellulose membrane (M 1 kDa) until the free halide ions were washed out and the purified HA was finally freeze-dried. The sample was left to equilibrate in the ambient laboratory atmosphere at about 25 °C, which resulted in the final equilibrium moisture content of about 8.4% by weight.

**Detailed discussion of SBPHA characteristics**

*Elemental composition and UV/Vis characteristics of SBPHA*

The elemental composition, atomic ratios and spectroscopic coefficients of SBPHA are presented in Table 1 and 2. As already emphasized in the paper, the elemental composition is expressed in atomic percent (at. %, equivalent to molar %), because, it better reflects the proportion of hydrogen in the structure of humic substances. The elemental composition and/or atomic ratios of peat HA were partially similar to that of peat humic acids from other regions of the world [1–3].

In the present study, the value of the $E_{ET}/E_{Bz}$ ratio for SBPHA was determined to ~ 0.7 (see Table 2). This result suggests that the substitution of aromatic rings with oxygen-containing functional groups was comparable with humic acids isolated from other caustobioliths (i.e. lignite and leonardite) [4, 5]. Another absorption coefficient described in the literature is the
The $E_2/E_4$ ratio (also referred to as $A_{265}/A_{465}$) which is frequently used as an indicator of the degree of aromaticity and average molecular weight, was utilized in this study for characterization of peat HA. We assume that the lower value of the $E_2/E_4$ ratio for SBPHA can be attributed to the higher average molecular weight rather than be related to highly differences in the aromaticity of the studied samples. In this study, the $SUVA_{254}$ obtained for original HA was 5.68. This value of the $SUVA_{254}$ is characteristic of humic acids from terrestrial origin [4, 6].

**FTIR characterization of SBPHA**

The FTIR spectra of SBPHA were obtained by means of an Attenuated Total Reflectance (ATR-FTIR) and, for comparison, also by Diffuse Reflectance Infrared Fourier Transform (DRIFT) technique using a Nicolet iS50 spectrometer. Method of ATR analysis is described in Materials and Methods. For the DRIFT analysis, approximately 2 mg of powdered HA sample was homogenized with 200 mg of KBr in an agate bowl. DRIFT spectrum was recorded over the 4000–400 cm$^{-1}$ range at a resolution of 4 cm$^{-1}$ and was the average of 512 scans. The infrared grade spectrum of annealed KBr was used as the background for DRIFT measurement.

Both FTIR spectra of SBPHA are presented in Figure S1. The comparison illustrates typical features of ATR spectra – linear dependence of penetration depth on frequency (affecting apparent intensity) and a slight shift of infrared bands. As far as the refractive index of SBPHA (and organic fractions) was not determined directly, the ATR spectra were not corrected and compared in the as-recorded form.

![Figure S1: ATR-FTIR and DRIFT spectrum of SBPHA](image-url)
The spectrum contains characteristic absorption bands typical for humic acids isolated from peat and coals [5, 7]. The DRIFT spectrum contains a sharp and intensive band at 1610 cm\(^{-1}\) corresponding to stretching C=C groups in aromatic rings. Another aromatic band is apparent at 1509 cm\(^{-1}\), due to the aromatic C=C stretching of lignin residues. Another significant band occurring at 840 cm\(^{-1}\) can be assigned to out-of-plane C–H deformation in aromatic rings with tri-substitution per ring. Absorption band at 1720 cm\(^{-1}\) corresponds to carboxylic groups, which are also indicated also by the broad band centered at about 2650 cm\(^{-1}\) resulting from the O–H stretching vibration modes of the hydrogen-bonded –COOH which forms dimers. The broad band centred at about 3225 cm\(^{-1}\) corresponds to the O–H stretching of various functional groups such as carboxylic, phenolic and alcoholic functional groups. The fingerprint region is characterized by an intensive bands at 1222 cm\(^{-1}\) and 1180 cm\(^{-1}\) corresponding to the C–O stretching and O–H bending of carboxylic groups, phenoxy structures and ethers, the C–O stretching of phenols and ethers. The spectrum contains an intensive band at 1080 cm\(^{-1}\) which can be attributed to the C–O–C vibration in aliphatic ethers, and to the C–O stretching of secondary alcohol moieties. The appearance of hydrocarbons is revealed in the 3000–2800 cm\(^{-1}\) zone of valence vibration. The absorption band at 2930 cm\(^{-1}\) was ascribed to asymmetric C–H stretching in methylene groups. From the ATR-FTIR spectra, the \(I_{CH2}/I_{CH3}\) and \(I_{Ar}/I_{COOH}\) ratios were calculated similarly to the extracted organic fractions (see Table 2). The values of both parameters were comparable to the last extracted organic fraction (MET6). This suggests that from the viewpoint of the structure of alkyl chains and the substitution of aromatic rings with carboxylic groups was practically identical for the parental HA and its last extracted fraction.

**Fluorescence spectroscopy**

The EEM contour map of the SBPHA is shown in Figure S2. The fluorescence domain is only located at excitation wavelengths in the ultraviolet region in the range 250–260 nm and at emission wavelengths in the range 480–500 nm. This fluorophore lies within the region referred to as the fulvic-like region, which is marked to as the A. Similarly to the two most polar organic fractions (PRO5 and MET6), A-type fluorophores were restricted to wavelengths in the range 255–265/495–505 nm, suggesting that all of them have similar structure of this fluorescence domain.
Figure S2: EEM spectrum of HA isolated from South Bohemian peat (SBPHA)

References

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Supplementary section 2:
Characterization of the solvents used in the polarity-based sequential extraction

Table S1: List of fundamental physicochemical properties of organic solvents used for extraction of organic fractions

| Organic solvent | Density (g mL⁻¹)¹ | Boiling point (°C)¹ | Dielectric constant¹ | Dipole moment¹ | Relative polarity index² |
|-----------------|-------------------|---------------------|----------------------|----------------|-------------------------|
| chloroform      | 1.498             | 61.20               | 4.80                 | 1.00           | 0.259                   |
| ethyl acetate   | 0.894             | 77.00               | 6.00                 | 1.78           | 0.228                   |
| acetone         | 0.786             | 56.20               | 21.00                | 2.85           | 0.355                   |
| acetonitrile    | 0.786             | 81.60               | 37.50                | 3.50           | 0.460                   |
| 1-propanol      | 0.803             | 97.00               | 22.00                | 1.68           | 0.617                   |
| methanol        | 0.791             | 64.60               | 33.00                | 1.60           | 0.762                   |

¹Properties of Solvents Used in Organic Chemistry. http://murov.info/orgsolvents.htm (accessed 23 October 1998).

²Reichardt, C.; Welton, T. Solvents and Solvent Effects in Organic Chemistry, 4th ed.; WILEY-VCH, Weinheim, 2010, p. 718.
Supplementary section 3:
Supplementary material to discussion of compositional and spectral characteristics of the obtained fractions

**Figure S3**: Van Krevelen diagram of organic fractions and original SBPHA

**Figure S4**: Deconvolution of the overlapping region of ATR-FTIR spectra at 1800–1500 cm\(^{-1}\). Positions of the bands were determined from the second-order derivative of ATR-FTIR spectra. Deconvolution was performed in OriginLab 8.1 software.
Table S2: Summary of fluorescence domains characteristic of humic substances and/or dissolved organic matter

| Fluorescence domains (fluorophores) | Excitation-emission wavelength pair | Classification          |
|--------------------------------------|-------------------------------------|-------------------------|
|                                      | Excitation (nm) | Emission (nm)             |                          |
| A                                    | 240–280          | 400–500                   | Fulvic-like\(^1\)       |
| C                                    | 300–380          | 400–500                   | Humic-like\(^1\)        |
| V                                    | 400–450          | 450–510                   | Humic-like\(^2\)        |
| M                                    | 290–310          | 370–410                   | Marine humic-like\(^1\) |
| B                                    | 270–280          | 300–315                   | Tyrosine-like\(^1\)     |
| T                                    | 270–280          | 345–360                   | Tryptophan-like\(^1\)   |
| H                                    | 250–320          | 300–380                   | Soluble microbial by-product-like\(^2,3\) |

\(^1\)Birdwell, J.E.; Engel, A.S. Characterization of dissolved organic matter in cave and spring waters using UV–Vis absorbance and fluorescence spectroscopy. *Org. Geochem.* **2010**, *41*, 270–280.

\(^2\)Doskočil, L.; Enev, V.; Pekař, M.; Wasserbauer, J. The spectrometric characterization of lipids extracted from lignite samples from various coal basins. *Org. Geochem.* **2016**, *95*, 34–40.

\(^3\)Chen, W.; Westerhoff, P., Leenheer J.A.; Booksh K. Fluorescence Excitation–Emission Matrix Regional Integration to Quantify Spectra for Dissolved Organic Matter. *Environ. Sci. Technol.* **2003**, *37*, 5701–5710.
Figure S5: EEM spectral cut-outs showing secondary fluorescence maxima
Figure S6: Liquid-state $^{13}$C NMR spectra of the studied organic fractions extracted from peat HA.