The new approach to assessing the qualitative composition of soil organic matter

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Abstract. There is an opinion [1] that both humic substances (HSs) and non-humic ones pass into alkaline extract from soil organic matter (SOM). Therefore, it became necessary to develop new approaches for isolation of HSs. In this publication, the motivation for new approaches for isolation of HSs, including melanins, from soil organic matter was provided based on a review of the scientific literature. These approaches include: (i) isolation of individual organic compounds, which are soluble in 90% dimethyl ketone (acetone) solution (specifically chlorins and carotenoids); (ii) isolation of HSs plus melanins and glomalins in 90% dimethyl ketone solution with HCl; (iii) sorption of HSs plus melanins and glomalins by a sorbent (in particular based on capronic acid); (iv) desorption of glomalins by phosphate buffer solutions with pH = 7; (v) desorption of HSs including melanins by phosphate buffer solutions with pH = 9, and 11, and 0.1 M NaOH solution with pH = 13. According to the existing classification, isolated HSs including melanins can be categorized as humic acids, but no fulvic acids have been found.

1. Introduction

Soil organic matter (SOM) is a very dynamic and heterogeneous complex system, which consists of specific substances (especially humic) and individual (non-specific) organic compounds (OCs), as well as products of interaction between each other and with the mineral part of the soil [2]. Humic substances (HSs), as follows from the scientific literature review [3], are organic natural dark-colored nitrogen-containing amorphous amphiphilic amphoterichigh-molecular stochastic redox-heteropolymers. The most important property of HSs is that they are colloids [3, 4]. It is believed [5, 6] that possible precursors of HSs are melanins (amorphous high-molecular heteropolymer dark-colored pigments), which are formed by prokaryotes, including actinomycetes, and fungi. The variety of starting monomers and the high activity of intermediates make the chemical composition of melanins vary and their polymer structure irregular [7, 8]. In SOM there are other specific organic substances: glomalins [9, 10], hydrophobins [11–13] and kerogen [14]. Individual organic compounds (IOCs) are another part of SOM in addition to HSs, which are not part of the HSs. One part of the IOCs is the components of organisms that lived on and/or in the soil, as well as their excreta, and the other part is the products of transformation of excreta and postmortem residues of biota [15]. The chemical composition of excreta and postmortem residues of biota entering the soil is very diverse [16, 17]. Individual organic compounds include: lipids
[15–19], pigments [20–25], etc., as well as particulate organic matter [26, 27]. High-molecular components of SOM (for example, HSs, melanins, lipids, biota litter, etc.) are gradually transformed by chemical and biochemical reactions into low-molecular components [15]. Low-molecular products of biochemical oxidative and hydrolytic destruction are rapidly updated (labile) organic material. Individual organic compounds of secondary origin can be found in the soil, in particular: phenolic compounds, amino acids, aliphatic acids, as well as oligo- and monosaccharides, including aminosaccharides and uronic acids [15–17]. Usually, to characterize SOM the qualitative composition methods are used, which are based on the isolation of so-called humic acids (HAs), fulvic acids (FAs) with aqueous alkaline or neutral salt solutions, and combinations of an aqueous base and pyrophosphate [16, 17, 28, 29]. Nevertheless, there is an opinion [1] that the isolation of the so-called HSs by any alkaline solutions and their subsequent fractionation is not correct.

In our opinion, the main method of studying the SOM qualitative composition should be based on the sequential isolation of certain groups of OCs.

Firstly, it is necessary to isolate IOCs from SOM, which are soluble in a 90% solution of dimethyl ketone (acetone). This group of OCs consists of chlorines (chlorophylls, pheophytins) and carotenoids, as well as some other OCs (perhaps hydrophobins).

Secondly, it is necessary to isolate “clean” HSs, including melanins. These specific substances do not need to be dissolved, they should be transferred to the liquid phase (more specifically, to a disperse medium) by creating certain conditions. To do this, it is important to remember that HSs and melanins are colloidal dispersions. It is known that HSs and melanins molecules have both hydrophilic and hydrophobic parts, i.e. the molecules of these substances are amphiphilic or diphilic [30]. Therefore, HSs and melanins are detergents and can spontaneously form structured colloidal micelles [31–34]. In turn, these spheroid structures of HSs can form aggregates resembling a bunch of grapes [16]. These specific substances should be regarded as supramolecular associations of self-assembling heterogeneous and relatively small molecules [35]. In structured colloidal micelles, the HSs and melanins molecules are bound to polynvalent metal ions [36]. It is assumed that in order to “disassemble” these structured colloidal micelles (for the phase change), it is necessary to “remove” polynvalent metal ions. For this purpose, Lewis bases should be added to a non-aqueous low-polar solution. These bases react with Lewis acids (metal ions), causing HSs micelles to decompose. During the “disassembly” of structured colloidal micelles of HSs, including melanins, molecules of solubilizates will be also released, which would not penetrate into the micelles, but would be fixed on their surface. Such substances can be glomalins. Glomalins are glycoproteins (proteins containing a carbohydrate component), or, more correctly, glycoconjugates. These compounds are complex hydrophobic OCs [9]. Glomalins are a product of arbuscular mycorrhizal fungi (AMF) of the order Glomales (Glomerales), hence the name “glomalins” [37]. Currently, there are a number of issues related to the study of glomalin. Modern methods for glomalins isolation, due to their similarity with the methods used for the HSs isolation, contribute to the isolation of both materials [38]. It is suggested that HSs with melanins can be separated from glomalines based on different surface-active properties or distinct values of the aggregative stability of these OCs.

Therefore, the purpose of this publication was to present new approaches to the isolation of “clean” HSs, including melanins, from SOM.

2. Objects and methods of research

2.1. Objects
Humus-accumulated horizons of soils of the forest-steppe zone were taken as study objects. The name, geographical location and a brief characteristic of the studied soils are shown in table 1. Geographical location of the study objects includes Central Russian Upland, Russian Federation, Belgorod oblast, State Natural Reserve “Belogor’e”.
Table 1. Name and geographical location of the studied soils.

| Horizon | Depth sampling, cm | Soil horizon | Soil name | GPS coordinates |
|---------|--------------------|--------------|-----------|-----------------|
| AY      | 0–22               | Umbric       | Grey (Greyzemic Phaeozem) on calcareous loess-like loam (oak wood) | N 50.60965° E 35.96760° |
| AEL     | 22–36              | Eluvial-Umbric | Dark-grey (Greyzemic Phaeozem) on calcareous loess-like loam (oak wood) | E 35.96760° |
| AU      | 0–15               | Mollic       | Mollic (eluvial) Magnobrucean on calcareous loess-like loam (oak wood) | E 36.30517° |
| AUe     | 15–32              | Mollic       | Eluvial-Magnobrucean on calcareous loess-like loam (oak wood) | E 36.30517° |
| AU1     | 0–17               | Chernic      | Chernic (eluvial) | E 36.5003° |
| AU2     | 17–50              | Chernic      | Chernic on calcareous loess-like loam (steppe) | E 36.05153° |

The average reaction of all soils was close to neutral (table 2). The values of total exchangeable bases in the humus horizons of the selected soils were characterized as high and very high. The horizons of the tested soils had an increased and high degree of base saturation. The values of loss-on-ignition were tested in series: grey soil < dark-grey soil < migrational-mycellary chernozem.

Table 2. Brief characteristics of the research objects.

| Horizon | pH (H₂O) | Hydrolytic acidity | Total exchangeable bases cmol/kg soil | Cation exchange capacity | Degree of base saturation % |
|---------|----------|--------------------|--------------------------------------|--------------------------|-----------------------------|
| AY      | 6.7      | 5.3                | 22.1                                 | 27.4                     | 80.7                        |
| AEL     | 6.3      | 4.8                | 23.2                                 | 28.0                     | 82.9                        |
| AU      | 6.5      | 3.5                | 27.2                                 | 30.7                     | 88.6                        |
| AUe     | 6.5      | 3.9                | 26.2                                 | 30.1                     | 87.0                        |
| AU1     | 6.6      | 1.2                | 44.4                                 | 45.6                     | 97.4                        |
| AU2     | 6.8      | 1.4                | 45.1                                 | 46.5                     | 97.0                        |
| Fst     | 0.31     | 64.35              | 30.94                                | 15.16                    | 0.92                        |
| F₀₅₈   | 3.11     | 3.11               | 4.12                                 | 3.79                     | 8.1                         |
| LSD₀₅₈ | –        | 0.66               | 5.84                                 | 7.06                     | –                           |

Table 2. (Continuation).

| Horizon | Hygroscopic moisture | Loss-on-ignition | Soil organic carbon | Degree of intramolecular oxidationb |
|---------|----------------------|------------------|---------------------|------------------------------------|
| AY      | 4.20                 | 8.53             | 3.64                | 2.74                               | –32.6                         |
| AEL     | 4.60                 | 8.78             | 2.24                | 2.85                               | 21.3                          |
| AU      | 4.49                 | 9.83             | 4.12                | 3.27                               | –25.9                         |
| AUe     | 5.10                 | 10.22            | 3.15                | 3.43                               | 8.1                           |
| AU1     | 5.18                 | 10.71            | 3.79                | 3.63                               | –4.5                          |
| AU2     | 5.24                 | 11.07            | 3.47                | 3.77                               | 8.0                           |
| Fst     | 2.42                 | 3.27             | 12.21               | 4.86                               | 76.88                         |
| F₀₅₈    | 3.11                 | 3.11             | 3.11                | 3.11                               | 3.11                          |
| LSD₀₅₈  | –                    | 0.280            | 0.090               | 0.087                              | 2.87                          |

a Fst – statistics, the Fisher criterion; F₀₅₈ – the Fisher critical values criterion for the significance level of 0.05; LSD₀₅₈ – the least significant digit for the significance level of 0.05.
b Degree of intramolecular oxidation of SOM (± d) is the calculated parameter.
The weighted average amount of CO\textsubscript{OX} and CO\textsubscript{CO2} (soil organic carbon) in all the tested objects was about 2.7 and 3.7\%, accordingly. Among the upper parts of humus horizons, AY horizon of the grey soil was the most reduced, and AU1 horizon of chernozem was the most oxidized, in accordance with the values of the degree of intramolecular oxidation. Among all objects, the AEL horizons were the most oxidized (table 2). It should be clarified that CO\textsubscript{OX} – chemical oxygen demand – is soil organic carbon (SOC), which was determined by the K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} oxidation [39], and CO\textsubscript{2} is the “actual” SOC that corresponds to the carbon determined on the basis of CO\textsubscript{2}.

2.2. Methods

Brief characteristics of the research objects were carried out using common methods [40]: the hydrogen ion exponent (pH\textsubscript{H\textsubscript{2}O}) of water suspension – by the potentiometric method, hydrolytic acidity (H) – by acid-base titration, total exchangeable bases (S) – by the Kappen’s method, cation exchange capacity (CEC) – by summation of hydrolytic acidity and total exchangeable bases (CEC = H + S), the degree of base saturation (V) – by the calculation method (V = \text{S} \cdot 100/(H + S)\textsuperscript{-1}), hygroscopic moisture (HM) – by thermostatic-weight method, loss-on-ignition (LOI) – by calcination in a muffle at 900 °C; CO\textsubscript{OX} (SOC)’ was determined from SOM oxidation by K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} [41]; CO\textsubscript{2} (SOC”) was evaluated on the LOI basis [42]: CO\textsubscript{2} = 0.405 \cdot LOI – 0.710; the degree of intramolecular oxidation (\pm d) was calculated by the following formula [43]: \pm d = (CO\textsubscript{2} – CO\textsubscript{OX}) \cdot 100 \cdot CO\textsubscript{CO2}\textsuperscript{-1}; organic carbon (CO\textsubscript{X}) of water solutions – by the method without evaporation and with using K\textsubscript{2}Cr\textsubscript{2}O\textsubscript{7} [44].

The test of the SOM qualitative composition included: (i) preliminary isolation of IOCs, which are soluble in 90% dimethyl ketone solution (specifically, chlorins and carotenoids); (ii) isolation of HSs with melanins and glomalins by organic solvent with Lewis bases; (iii) separation of HSs including melanins from glomalins by desorption from a sorbent based on different aggregative stability.

Chlorins, carotenoids and some other compounds were isolated from SOM by a 90% dimethyl ketone solution. Two-isolation was used [25]. The content of photosynthetic pigments was determined using a VIS-spectrophotometer (model UV–9600, Rayleigh, Beijing, China). The concentration of chlorophylls a and b (Chl-a and Chl-b), pheophytins (Ph) and carotenoids (Car) in a dimethyl ketone (acetone) solution (mcg/l) was calculated using the following formulas according to GOST 17.1.4.02-90:

\[
C\text{chl-a} = (2.44 \cdot (D_{664} - D_{664}^4)/D_{664}) \cdot (11.85D_{664} - 1.54D_{647} - 0.08D_{630})
\]

\[
C\text{chl-b} = 21.03D_{647} - 5.43D_{664} - 2.66D_{630}
\]

\[
C\text{ph} = (2.44 \cdot (1.7D_{664}^4 - D_{664})/D_{664}) \cdot (11.85D_{664} - 1.54D_{647} - 0.08D_{630})
\]

\[
C\text{car} = 4D_{480}
\]

\[
I_{430/664} = D_{430}/D_{664}
\]

where: $D_{664}$ and $D_{664}^4$ – optical densities of solutions in Bells at $\lambda = 664$ nm before and after its acidification; $D_{430}$, $D_{480}$, $D_{630}$ and $D_{647}$ – optical densities of solutions in Bells at $\lambda = 430$, 480, 630 and 647 nm, respectively; $I_{430/664}$ – pigmentation index.

When calculating the concentration, the value of the solution (Vs, ml) and the cuvette length (l, cm) were taken into account. All optical densities included in the formulas were taken with the correction equal to the optical density at $\lambda = 750$ nm. This correction was subtracted from the measured optical density.

Secondly, glomalins and HSs, including eu- and allomelanins, were isolated by a 90% dimethyl ketone solution with HCl. It should be noted that chloride-ions are Lewis bases.

The mixture containing, as expected, both HSs with melanins and glomalins was diluted twice with distilled water and mixed with a sorbent (capronic acid). Capronic acid was obtained in a laboratory according to the recipe [45]. Desorption was performed using a series of phosphate buffer solutions with pH = 3, 5, 7, 9 and 11, and 0.1 M solution NaOH (pH = 13). Solutions with pH = 3 and 5 did not isolate...
OCs. The solution with pH = 7 presumably desorbed glomalins. Solutions with pH 9, 11 and 13 isolated HSs with melanins. These solutions were then acidified with a 0.1 M HCl solution to pH ~ 2. As a result, HSs lost their aggregate stability and precipitated. The precipitate of HSs was washed with distilled water and transferred to the liquid phase by a 0.02 M NaOH solution. Isolated HSs can be categorized as HAs according to the existing classification. No fulvic acids were detected. This confirms that FAs are artifacts [46]. Thereafter HSs with melanins has been purified by a strong cationite (Purolite C-100). In turn, glomalins were purified from mineral salts by dialysis through a cellophane membrane.

The composition of a complex mixture of OCs can be characterized by the ratio of absorbance at λ = 465 nm and at λ = 665 nm [47]. For this aim, optical indexes Q_{465} are usually calculated. Optical indexes Q_{465} are often used to evaluate the HSs quality [16, 48–50]. As absorbance at λ = 465 nm occurs due to smaller molecules, and at λ = 665 nm due to larger molecules. Consequently, the Q_{465} ratio will be larger for FAs with low molecular weight and smaller for HAs with greater molecular weight studies [49]. Q_{465} was used as well. Optical characteristics were determined in pre-neutralized (up to pH ~ 7) tested solutions.

The 3-fold replication was used for all tests. For statistical processing of the experimental data, dispersion analysis was used [51, 52]. If the data was obtained as a percentage, then the Fisher’s angular transformation was used before performing the variance analysis. In addition, the weighted average values were calculated taking into account different depths of the soil horizons.

3. Results and discussion

As our experimental data showed, chlorophylls a and b and pheophytin were only present in the SOM formed under forest, and carotenoids were present in the SOM of all objects (table 3). Thus, chlorophylls a and b were in the top part of the humus horizons of gray (AY) and dark-gray (AU) soils, and pheophytins – in the top part of the humus horizons of gray soil only. In addition, the pheophitin content was higher than the total content of chlorophylls a and b. Apparently, the combination of a relatively high hydrolytic acidity value and the highest reduction of SOM led to the transformation of chlorophylls into pheophytines. In our opinion, the presence of a steppe mat on the surface of virgin chernozems prevented the entry of green parts of plants into the soil, so chlorophylls were absent in both AU1 and AU2 horizons. So the plant litter remained on the surface of soils and was exposed to UV solar radiation, as a result of which the main part of the plant litter of chlorophylls was destroyed.

It is known [24] that there is a certain reserve of chlorins in soils that are capable of being preserved without destruction. The content of chlorophylls in soils is connected to their water regime [53]. Carotenoids and chlorines can be preserved in buried soils for up to several thousand years [20, 23]. The decomposition of 14Clabeled chlorophylls and β-carotene in soil is slower compared to the decomposition of glucose, hemicellulose and cellulose [54]. At the same time, under aerobic conditions of the forest floor, carotenoids are less resistant to decomposition than pheophytin a, the dominant chlorophyll derivative in soils [55].

As shown by a series of model experiments [56], the destruction of chlorophylls takes place in two stages: the first, very fast stage occurs in the tissues of the plants themselves, the second, slower, in soils under the influence of soil biota. The intensity of microbial decomposition is regulated by conditions such as soil acidity, humidity and temperature, as well as the presence of toxic substances, etc. Microorganisms decomposed both chlorophylls a and b within two to four months in field soils; chlorophyll a was attacked most. Of the chlorophyll-type compounds, pheophytin, the most closely related chlorophyll derivative, was the most resisted to decomposition [56]. The weighted average amount of carotenoids in all tested soil horizons was about 1.8 g kg⁻¹ of soil. In the case of grey soil and chernozem, the content of carotenoids in the upper part of the humus horizon was significantly higher than in the lower part (table 3). At the same time, the content of carotenoids in the lower part of the dark-grey soil humus horizon was significantly higher than in the upper part. These facts can obviously be explained by the specific distribution of the plant roots enriched with carotenoids. Carotenoids are the most widely distributed class of natural pigments of isoprenoid nature, they can be synthesized de novo by prokaryotes, algae, fungi, higher plants and other living organisms [7, 57]. It should be noted that most
plants are characterized by low levels of carotenoids in the roots [58]. The high antioxidant activity of carotenoids [59] contributes, in our opinion, to the preservation of these pigments in the SOM composition. Besides, as it was experimentally determined [60], chlorines and carotenoids are able to solubilize in structured colloidal micelles of HSs. This phenomenon can serve as an additional explanation for the long-term presence of photosynthetic pigments in SOM.

Table 3. Content of chlorophyll $a$, chlorophyll $b$, pheophytins and carotenoids in SOM of the test objects.

| Horizon          | Chlorophyll $a$ | Chlorophyll $b$ | Pheophytins | Carotenoids |
|------------------|-----------------|-----------------|--------------|-------------|
| Grey soil        |                 |                 |              |             |
| AY               | 6.5             | 4.7             | 17.7         | 2.7         |
| AEL              | 0               | 0               | 0            | 1.9         |
| Dark-grey soil   |                 |                 |              |             |
| AU               | 12.6            | 0.6             | 0            | 1.3         |
| AUe              | 0               | 0               | 0            | 6.8         |
| Migrational-mycellary chernozem |         |                 |              |             |
| AU1              | 0               | 0               | 0            | 1.3         |
| AU2              | 0               | 0               | 0            | 0.6         |
| Fst              | 55.54           | 224.63          | –            | 149.01      |
| F05              | 7.71            | 7.71            | –            | 3.11        |
| LSD05            | 2.28            | 0.76            | –            | 0.56        |

As follows from the obtained optical indices ($Q_{4/6}$) of OCs solutions (table 4), the values of $Q_{4/6}$ were the highest in the fractions desorbed by a solution with pH = 7, and the lowest in the fractions desorbed with a solution with pH = 13. The values of the optical indices of the solution decreased with increasing alkalinity of desorption solutions. There is a common notion that the lower the optical indices ($Q_{4/6}$) of HSs, the higher the condensation degree of molecules (the dominance of aromatic structures over aliphatic ones) would be [16, 48], the higher the molecular weight is [49]. It is known [16] that the values of $Q_{4/6}$ for HAs conform to: about 5.0 for podzolic soils, 3.5 for dark-gray soils, and 3.0–3.5 for chernozems, while the $Q_{4/6}$ values of FAs from different soil types ranged from 6.0 to 8.5.

Table 4. Optical indexes $Q_{4/6}$ of organic compounds solutions.

| Horizon          | Optical indexes $Q_{4/6}$ |
|------------------|---------------------------|
|                  | 1st (pH = 7)              | 2nd (pH = 9) | 3rd (pH = 11) | 4th (pH = 13) |
|                  | Fractions of organic compounds (with different pH of desorption solutions) |
| Grey soil        |                           |             |               |               |
| AY               | 7.8                        | 4.5         | 3.6           | 2.6           |
| AEL              | 6.2                        | 4.6         | 3.5           | 2.7           |
| Dark-grey soil   |                           |             |               |               |
| AU               | 6.8                        | 4.1         | 3.1           | 2.8           |
| AUe              | 7.0                        | 4.2         | 2.9           | 3.4           |
| Migrational-mycellary chernozem |         |                 |              |             |
| AU1              | 7.5                        | 4.2         | 3.1           | 2.6           |
| AU2              | 9.5                        | 5.0         | 4.3           | 3.1           |
| Fst              | 5.32                       | 1.40        | 5.00          | 2.86          |
| F05              | 3.11                       | 3.11        | 3.11          | 3.11          |
| LSD05            | 1.53                       | –           | 0.70          | –             |

The optical indices values of the 1st fractions with pH = 7 corresponded to the values of FAs. Organic compounds of these fractions had an orange-yellow color as FAs. But in contrast to FAs, these organic compounds were not precipitated by KAl(SO$_4$)$_2$. In addition, these fractions were tested by qualitative reactions. It was found that protein tests (xanthoproteic and biuret reactions and reaction with ninhydrin),
as well as carbohydrates test (Molish reaction with α-naphthol) gave positive results. These investigations have confirmed that OCS of fractions with pH = 7 were glomalins (glycoproteins).

The content of glomalins carbon (C_{OX}), which was determined by K2Cr2O7 oxidation of OCS, was the same in all horizons (table 5). The weighted average of glomalin carbon (C_{OX}) in all tested objects was about 3.3 g·kg⁻¹ of soil. The relative content of glomalin carbon to SOC' (which was determined based on the oxidation of SOM by K2Cr2O7 too) was in the range from 8.5 to 14.1%.

The high content of glomalins is observed mainly due to the abundance of AMF hyphae in soils, the length of which can be up to 100 m·cm⁻³ [61], and the slow rate of destruction of these glycoproteins – from 7–42 to 100 years [10, 62]. A common pattern was found between the synthesis of glomalins and melanins [63]. Glomalins in SOM are present in large quantities (usually 2–15 g·kg⁻¹ and even more than 60 g·kg⁻¹) in a variety of soils (both acidic and calcareous) [9]. In our case, the number of glomalins isolated without HSs, including melanins, was not high (table 5).

| Horizon          | Organic carbon (C_{OX}) of fractions (with different pH of desorption solutions) | 1st (pH = 7) | 2nd (pH = 9) | 3rd (pH = 11) | 4th (pH = 13) | Sum 2nd–4th |
|------------------|--------------------------------------------------------------------------------|--------------|--------------|--------------|--------------|-------------|
|                  | Glomalins                                                                   | g·kg⁻¹ soil | % to soil C_{OX} | g·kg⁻¹ soil | % to soil C_{OX} | g·kg⁻¹ soil | % to soil C_{OX} | g·kg⁻¹ soil | % to soil C_{OX} | g·kg⁻¹ soil | % to soil C_{OX} |
| Grey soil        |                                                                             |              |               |             |               |             |               |             |               |             |               |
| AY               |                                                                             | 3.36         | 9.2           | 4.64        | 12.7          | 5.08        | 14.0          | 7.88        | 21.6          | 17.60        | 48.4          |
| AEL              |                                                                             | 3.16         | 14.1          | 4.76        | 21.3          | 4.92        | 22.0          | 7.08        | 31.6          | 16.76        | 74.8          |
| AU               |                                                                             | 3.52         | 8.5           | 4.60        | 11.2          | 5.32        | 12.9          | 6.72        | 16.3          | 16.64        | 40.4          |
| AUe              |                                                                             | 3.16         | 10.0          | 4.48        | 14.2          | 6.36        | 20.2          | 9.00        | 28.6          | 19.84        | 63.0          |
| Dark-grey soil   |                                                                             |              |               |             |               |             |               |             |               |             |               |
| AU1              |                                                                             | 3.36         | 8.9           | 4.64        | 12.2          | 5.08        | 13.4          | 7.88        | 20.8          | 17.60        | 46.4          |
| AU2              |                                                                             | 3.16         | 9.1           | 4.76        | 13.7          | 4.92        | 14.2          | 7.08        | 20.4          | 16.76        | 48.3          |
| Fst              |                                                                             | 0.48         | 8.89          | 0.12        | 13.14         | 2.47        | 12.78         | 2.70        | 13.41         | 1.09         | 12.06         |
| Fos              |                                                                             | 3.11         | 3.11          | 3.11        | 3.11          | 3.11        | 3.11          | 3.11        | 3.11          | 3.11         | 3.11          |
| LSD05            |                                                                             | –            | 0.37          | –           | 0.56          | –           | 0.65          | –           | 1.03          | –            | 4.49          |

Isolated HSs with melanins according to the existing classification, which could be categorized as HAs and FAs, were not found. The content of HSs was estimated by C_{OX} that was determined by K2Cr2O7 oxidation of HSs. The amounts of HSs carbon (g·kg⁻¹ soil) was the same in different objects within each fraction, which was desorbed by one or another alkaline solution (table 5). It was identified that as pH values of alkaline solutions increased, the number of HSs passing into these solutions also increased. The weighted average amount of HSs carbon (C_{OX}) in the fractions with pH ~ 9, pH ~ 11 and pH ~ 13 was 4.7, 5.2 and 7.6 g·kg⁻¹ of soil, accordingly. The relative content of HSs carbon (C_{OX}) to SOC’ was in the range from 11.2 to 21.3%, from 12.9 to 22.0% and from 16.3 to 31.6%, correspondingly. The weighted average total amount of HSs carbon (C_{OX}) isolated from all the tested horizons by three alkaline solutions was 17.5 g·kg⁻¹ of soil. The relative total content of HSs carbon (C_{OX}) to SOC’ was in the range from 40.4 to 74.8%. A trend was revealed – the content of HSs in the lower thickness of the tested horizons of all soils was higher than in the top one. In the case of forest soils, the difference between the upper and lower thickness of the tested horizons was the most distinct. The observed phenomenon was associated both with the difference in the soil carbon (C_{OX}) content and with the pedogenic process peculiarities that are different for steppe and forest soils.

Table 5. The content of glomalins and humic substances including melanins.
4. Conclusions
Thus, new approaches made it possible to develop a scheme for evaluating the qualitative composition of SOM based on the sequential isolation of different groups of organic compounds. This scheme includes: (i) preliminary isolation of IOCs (specifically chlorins and carotenoids) by a 90% dimethyl ketone solution; (ii) isolation of HSs with melamins and glomalins by a 90% dimethyl ketone solution with HCl; (iii) separation of HSs including melamins from glomalins by desorption from the sorbent based on different aggregative stability.

As it was revealed, chlorophylls a and b and pheophytin were present only in the organic matter of the upper layer of forest soils and carotenoids were present in the SOM of all studied objects. The weighted average amount of carotenoids in all tested soil horizons was about 1.8 g·kg⁻¹ of soil.

Humic substances and glomalins were isolated together, but then, these substances were separated by the sorption-desorption method. The glomalins content in all the tested horizons was the same. The weighted average amount of glomalins carbon (C_{OX}) in all objects was about 3.3 g·kg⁻¹ of soil. The relative content of glomalins carbon (C_{OX}) to SOC was in the range from 8.5 to 14.1%. Isolated HSs by a dimethyl ketone (acetone) solution with HCl can be categorized as HAs, but FAs were not found. The amounts of HSs carbon (C_{OX}) isolated from all the tested horizons by three alkaline solutions were the same in each fraction. The weighted average amount of HSs carbon (C_{OX}) in these fractions was 4.7, 5.2 and 7.6 g·kg⁻¹ of soil. The weighted average total amount of HSs carbon (C_{OX}) was 17.5 g·kg⁻¹ of soil. The relative total content of HSs carbon (C_{OX}) to SOC was in the range from 40.4 to 74.8%.

Uncertainties
The authors understand that the new scheme of HSs isolation from OM of natural objects is not yet fully worked out, that it is only the first approximation for decision of problems, which are connected with biochemistry of SOM.

Acknowledgments
This work was supported by Russian Foundation for Basic Research (grant no. 19-29-05243).

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