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Antioxidant Activity and Bioavailability of Humic Substances of Low-Mineralized Sulphide Mud

Zh. K. Kairbekov,[1,*] S. M. Suimbaeva,[1,*] I. M. Dzheldybaeva,[1] M. Z. Esenalieva,[1] A. Zh. Kairbekov[1] and D. Z. Abil'mazhinova[2]

Abstract

Humic substances (HS) are natural compounds, the formation of which occurs in natural environments as a result of chemical and biological decomposition. HS accounts for 60% to 80% of organic matter in soil and aquatic environment. Therefore, we obtained samples of HS isolated from low-mineralized sulphide mud deposits of Tuzkol Lake (Republic of Kazakhstan) were obtained. HS samples have been characterized by instrumental methods: EPR spectroscopy and amperometry. Analysis of the concentration of paramagnetic centers shows that the minimum number of free radicals contains molecules of fulvic acids, and the maximum number of free radicals contains molecules of humic acids (HA) of peloids. The antioxidant activity and bioavailability of humic preparations in the form of sodium humates depending on the concentration of HS have been investigated. The information obtained indicates that HS have antioxidant activity and bioavailability, the value of which increases when the concentration of the solution grows. Dialysis of silver humate has been carried out in the presence of dimethyl sulfoxide (DMSO) at concentrations of 0.1; 1.0; 5.0 and 10% w/w. For HA and silver humate, a 10% concentration of DMSO is optimal, which allows a maximum yield of peloid preparations and a 1.6-fold increase in permeability. Dialysis of silver humate is 1.2 times more intensive than dialysis of HA. It has been shown that the change in the total content of antioxidants in various fractions of humic substances increases in the series; fulvic - humic – gimatomelanic acids.

Keywords: Low-mineralized silt sulphide mud (peloid); Fulvic acids; Gimatomelanic acids; Humic acids; Antioxidant activity; bioavailability, dialysis.

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1. Introduction

Access to clean and safe drinking water is a fundamental requirement for human health and well-being. However, the presence of contaminants, such as humic acid, can pose significant challenges to achieving this goal. Humic acid, a natural organic compound derived from the decomposition of plant and animal matter, is a common pollutant found in water sources worldwide. It not only imparts undesirable taste, odor, and color to drinking water but also has the potential to react with disinfectants and form harmful byproducts.[1,2]

The removal of humic acid from drinking water has become a critical area of research and development in the field of water purification. Traditional water treatment processes, including coagulation, sedimentation, and filtration, often face limitations in effectively removing humic acids due to their complex chemical structure and stability in aqueous environments.[3-6] Therefore, there is a pressing need for innovative and efficient techniques to tackle this persistent water quality issue.

Humic acids are natural high-molecular compounds of irregular structure contained in peat, brown and oxidized coal, peloid, bottom sediments. The HA obtained from different sources differ in elemental composition, condensation degree, substitution of aromatic nuclei, ratio of hydrophilic and hydrophobic fragments. The composition of HA macromolecules includes various functional groups: carbonyl, carboxyl, hydroxyl. Due to the uniqueness of its structure, HA are able to enter into redox reactions, complexation and ion exchange reactions with metal cations, which in turn determines a wide range of their applications.[7-11]

In scientific terms, the redox properties of humic substances of soils, peat, brown coals, peloid, obtained by various chemical and physicochemical methods, have been the objects of research for a long time. However, the redox

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[1] *Al-Farabi Kazakh National University, Research Institute of New Chemical Technologies and Materials, Al-Farabi str 71, Almaty,050000, Republic of Kazakhstan.*

[2] *Department of Chemistry, Faculty of Natural Science, Kazakh National Woman's Teacher Training University, Aitekebi Street 99, Almaty 700420, Kazakhstan.*

**Email:* suimbayeva@gmail.com (S. Suimbaeva)

properties of humic substances isolated from peloid obtained from low-mineralized slit sulphide mud deposits of Kazakhstan for their use in pharmaceuticals have not been practically studied.

Peloids are complex substances formed in natural conditions of biocenoses due to the activity of hydrogeological processes. The formation of therapeutic mud occurs under the effect of a complex of natural factors. The most common peloid are formed by sedimentation in water bodies and swamps.[12] The annual decline in peloid reserves is explained not only by their constant extraction, but also by the degradation of mud deposits due to environmental pollution. Typically, deposits of therapeutic mud are contaminated with solid metals.[13]

Slit sulphide mud is characterized by a low degree of mineralization and is considered as a source of ecologically clean natural medicinal substances. Research in the development of new biologically active compounds has shown that humic substances of various origins have reparative, immunomodulating, anti-inflammatory, antioxidant effects, and are a universal matrix that allows the introduction of the necessary trace elements into the human body in the active form.[14-18]

A number of foreign scientists (Germany, Canada, Czech Republic, Russia) have proved the positive effect of HS on the physiological condition of individuals. HS are not real alternatives to strong traditional therapeutics. However, HS are also environmental chemicals. Due to the low-molecular masses of their building blocks, they appear to be capable to easily pass biomembranes.[19,20]

Along with the study of the properties of synthetic drugs in recent years, research and experimental work on the development of technologies for the production and use of natural antioxidants (AO), as fewer toxic compounds, and humic substances contained in therapeutic mud are gaining more practical importance.[21] The priority task of pharmaceuticals at present is the creation of innovative medicinal substances based on domestic natural raw materials that can be used in the production of modern competitive pharmaceutical products. From this point of view, humic substances are considered as promising, since they can reach the therapeutic effect at the subcellular and molecular level. The pharmaceutical use of humic substances of peloid formed under reducing conditions with the participation of sulfate-reducing microorganisms is of interest, since theses microorganisms affect their structure and chemical properties which determines the biological activity of compounds.[22-27] In this regard, the study of antioxidant activity and bioavailability of humic substances from peloid is very important.

This article contains the results of experimental data on the study of antioxidant properties and bioavailability of humic substances isolated from their low-mineralized silt sulfide therapeutic mud of Tuzkol Lake.

2. Experimental part

Tuzkol Lake is located in Rayymbek district of Almaty region, 330 km south-east of Almaty and 35 km north-east of the administrative center of Narynkol. The maximum depth of the lake reaches 0.9 m (average – 0.5 m), length – 6.3 km, maximum width – 2.8 km. The characteristics of the mud sample of Tuzkol Lake deposit are given in work.[11] Visually, the mud looks like a homogeneous soft-plastic mass of dark gray color with layers of black, with the smell of hydrogen sulfide and with a small sediment of liquid phase.

The average moisture content of mud is 48.0%, volume weight is 1.73 g/cm^3, shear resistance is 2500 Pa at a rate of 1500-1400 Pa. The heat capacity, depending on the value of moisture, is small and is 0.60 Calorie/Gram· Celsius, the reaction of the medium is neutral (pH – 7.75), the density is 0.048 g/cm^3. Contamination with mineral particles with a size of 0.25-5.0 mm – 0.20% while the norm is not more than 3%; mineral inclusions with a size of more than 5 mm are absent. Active ingredients in significant quantities have been detected in the mud: hydrogen sulfide – 0.11%, mobile iron – 0.35%, hydrotroillite – 0.20%. The peloid of Tuzkol Lake deposit belong to high-quality medium-sulphide, slightly alkaline slit mud.

The presence of paramagnetic centers (PMC) has been determined by *JEOL* JES-FA200 EPR spectrometer under normal conditions (T = 26 °C, Patm), with an operating frequency of 9.3 GHz at a modulation current of 0.10A. The value of the modulation current was 0.75 A. The concentration of PMC of free radicals have been obtained by comparing the spectral areas of the test sample with the spectral area of the point standard, calibrated according to the standard diphenylpicryl hydrazyl (DPPH). As a standard, DPPH $(C_6H_5)_2–N–N–C_6H_5\ (NO_3)_2$, consisting of 100% radicals, has been used, and in which 3.5 VE_2 (EPR-spectrum interval) accounts for 4.8-1015 PMCs. The intensity of the resonance line has been determined by the area under the absorption curves.

The total antioxidant activity of humic substances was evaluated on the chromatograph TsvetYauza-01-AA by amperometric method,[28] which is based on measuring the strength of the electric current arising from the oxidation of antioxidant molecules on the surface of the working electrode of the analyzer at a certain potential, after amplification of the converted into a digital signal. According to the results obtained, a calibration graph showing the dependence of the peak area (signal value) on the concentrations of quercetin have been obtained, for which the signals of standard solutions of quercetin have been sequentially recorded in order of increasing their concentrations.

For the preparation of the analyzed samples, the humic samples were crushed to the size of particles passing through a 1 mm diameter sieve. Accurately weighed (about 1g) crushed sample was placed in a 100 cm^3 flask, with addition of 70% ethyl alcohol and then shook on a stirring device for 1h. Then the contents of the flask were filtered through a paper filter into a 100 cm^3 volumetric flask, the filter was washed with ethyl alcohol and the filtrate was adjusted to the required amount by adding ethyl alcohol. The filtrate was diluted with bidistilled water when necessary.

Registration and mathematical processing of the analysis results was carried out using a universal computer system for information collecting and processing "*Z-lab*" and "Data processing system AD&KD".

Calculation of the mass concentration of HA antioxidants (X, mg/dm^3) was carried out by formula (1):

$$X = X_r N \qquad (1)$$

where, X_r is the mass concentration of antioxidants according to a graduated graph, mg/dm^3; N is the dilution factor of the analyzed sample.

In case of analysis of solid samples, the mass fraction of antioxidants, mg/g, is calculated by formula (2):

$$X = \frac{X_r V_n N}{m_n \cdot 1000} \qquad (2)$$

where, X_r is the mass concentration of antioxidants according to a graduated graph, mg/dm^3; V is the volume of the solution (extract) of the analyzed sample, cm^3; m_n *is* the weight of the analyzed substance, g; N is the dilution ratio of the analyzed sample.

The arithmetic mean of the results of two parallel determinations was taken as the measurement result.

Determination of the bioavailability of HS preparations have been carried out according to the following procedure: HS solutions of the same volume (2 ml) and different concentrations were placed in a dialyzer, which was immersed in a dialysate cup containing 10 ml of water (optimal volumes were determined experimentally). After 1 hour, the optical density of the dialysate was determined on a FEK-56M device at a wavelength of 400 nm, in 5 mm thick cuvettes.

3. Result and discussion

For the destruction of mineral complexes and the removal of sulfides, carbonates and other mineral components, native dirt was treated with a 2M solution of hydrochloric acid without thermal effects in order to prevent the destruction of organic substances. The production of cleaner preparations was carried out by extraction of specific organic substances with 0.5 M sodium hydroxide solution in the ratio of peloid: solvent = 1:10 no more than three times, since in subsequent portions the number of mineral components increased significantly. At this stage, an extract containing the sum of fulvic, hymatomelanic and humic acids was obtained. For further fractionation of specific organic substances, the precipitate was filtered and a 50% solution of sulfuric acid was added to the filtrate to pH = 1.0 (according to the universal indicator). The liquid from the precipitate was decanted and filtered. The fraction of fulvic acids was isolated according to the method.[21] Consequently, the filter residue was washed with water until negative reactions to sulfate ions and exhaustive extraction with ethanol until pale yellow staining of the extractant (gimatomelanic acid fraction) have been carried out. The residue on the filter after alcohol extraction is humic acids, which make up the basic part of humic substances. It was dissolved with 0.02 M sodium hydroxide solution, then precipitated with sulfuric acid. In order to obtain a low-ash preparation, the reprecipitation was carried out twice, after which the alkaline solution was passed through the cation exchanger and dried. To obtain solutions of humic substances of peloid, accurate weights of individual fractions of humic substances were filled with a certain amount of 0.05 M NaOH. The flask with the preparations was placed in a water bath, left for a day until the substances were completely dissolved. One time filtered through a paper filter (white tape), adjusted pH to 7.4, then diluted with purified water to 100 ml.

The isolated humic substances were investigated by EPR spectroscopy. From the obtained data (Table 1) it follows that HS is characterized by the presence of PMC with a g-factor with value higher than 2.00, which indicates the presence of free radicals in HS molecules due to a strongly delocalized electron cloud. Humic substances, being polyfunctional compounds containing both electron donor and electron acceptor substituents, are capable of forming complexes with charge transfer, which leads to the appearance of two unpaired electrons. The conducted experiments confirm the direct dependence of the intensity of the free electron signal on the magnitude of the interfacing systems.

Calculations of the integral intensity of the absorption lines made it possible to estimate the concentrations of unpaired electrons in the peloid HS samples. On the spectra of the studied samples, one intensive wide line can be identified with g= 2.0018 (g is the factor of spectroscopic splitting factor of Lande), which indicates the obvious presence of free radicals in the structure of the HS, and the quantitative content of $3.75*10^{18}$ spins/g allows predicting a good prospect of using humic acids of peloid of the Tuzkol deposit as natural antioxidants with high bioactivity. The presence of EPR signals indicates that humic substances are polymers with a well-developed system of conjugated double bonds. The concentration of paramagnetic centers was calculated from the known concentration of coal pitch spins, which is $3,66*10^{15}$ spins/g. Analysis of the concentration of paramagnetic centers (Table 1) shows that the minimum number of free radicals contains fulvic acid molecules of peloids. The concentration of paramagnetic centers in gimatomelanic acids exceeds 13

Table 1. Results of EPR spectroscopy of HS of peloid.

Sample No.	Sample Name	Free radical concentration, N, spins/g	EPR line width, ΔH, mT	g-factor
1	Fulvic Acid	0.09×10^{18}	0.616	2.0021
2	Gimatomelanic Acid	1.2×10^{18}	0.613	2.0019
3	Humic acid	3.75×10^{18}	0.617	2.0018

times and reaches the maximum value in humic acids, where their concentration in comparison with fulvic acids increases by 42 times. Low concentrations of paramagnetic centers in the gimatomelanic and fulvic acids of peloids indicate the probable possibility of stabilization of particles and are also characterized by less condensed structures, that is, lower content of aromatic structures. Gimatomelanic acids, like carotenoids and tocopherol, appear to be “traps” for radicals. These substances, reacting with R˙ or ROO˙ radicals, break the oxidation chains and inhibit lipid peroxidation. High contents of paramagnetic centers in humic acids of peloids characterize the presence of free radicals in the molecule, since these compounds are characterized by a high content of the aromatic component. Probably, these radicals are not "traps", but electron donors.

One of the methods that allow directly measuring the total content of antioxidants in the sample is amperometric,[27] in which the electric current arising from the electrochemical oxidation of the test substance on the surface of the working electrode is measured at a certain potential.

The average arithmetic value of five measurements (relative mean square deviation of not more than 5%) is taken as the result, and a calibration graph is plotted (Fig. 1). In the studied HS samples, the mass concentration of antioxidants equivalent to quercetin was determined according to the calibration graph of quercetin by calculating the peak area on the chromatograms obtained on the TsvetYauza-01-AA chromatograph with an amperometric detector. The total antioxidant content was determined in HS of peloid solutions at concentrations (wt %): 0.001; 0.010; 0.100 and 1.000.

The data presented in Table 2 illustrates that the total amount of antioxidants in humic substances gradually increases with their concentration.

From the data presented in Table 2 it can be seen that with increasing the concentration of antioxidants there is a consistent increase in the value of their total content in humic substances, and the highest value of the content of antioxidants compared to fulvic acids was observed in gimatomelanic and humic acids. At a concentration of 0.001% in humic acids, the content of antioxidants is 2 times less, in fulvic acids – 9 times less, compared to the concentration of antioxidants in gimatomelanic acids. With an increase in the concentrations of solutions of humic substances, an increase in the total content of antioxidants in the studied fractions was observed. In gimatomelanic acids in the concentration range of 0.001-0.01%, the content of antioxidants increased by 14 times, in fulvic acids by 34 times and in humic acids by 10 times. With a further increase in the concentration of humic substances

Table 2. Quantification of humic substances of peloid by the total content of antioxidants.

Indicators	Total antioxidant capacity (TAC), 10^{-2} mg/ml			
Solution concentration (wt.%)	0.001	0.010	0.100	1.000
Fulvic acid	0.02	0.67	2.52	29.67
Gimatomelanic acid	0.18	2.45	11.48	97.71
Humic acid	0.09	0.93	5.37	49.81

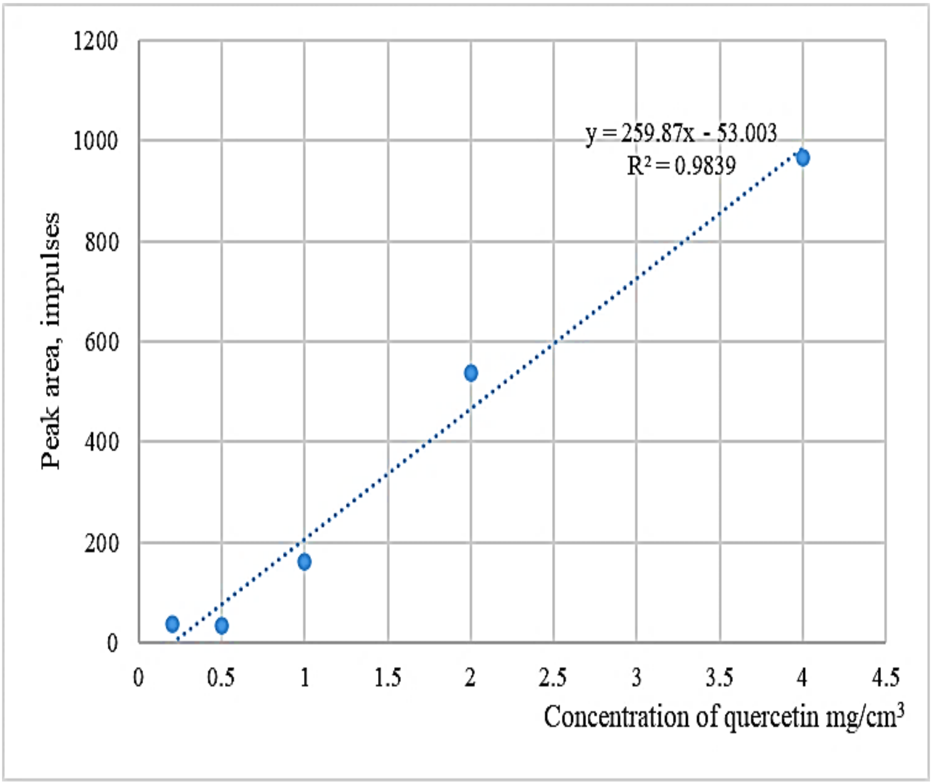

Fig. 1 Dependence of peak area from quercetin concentration.

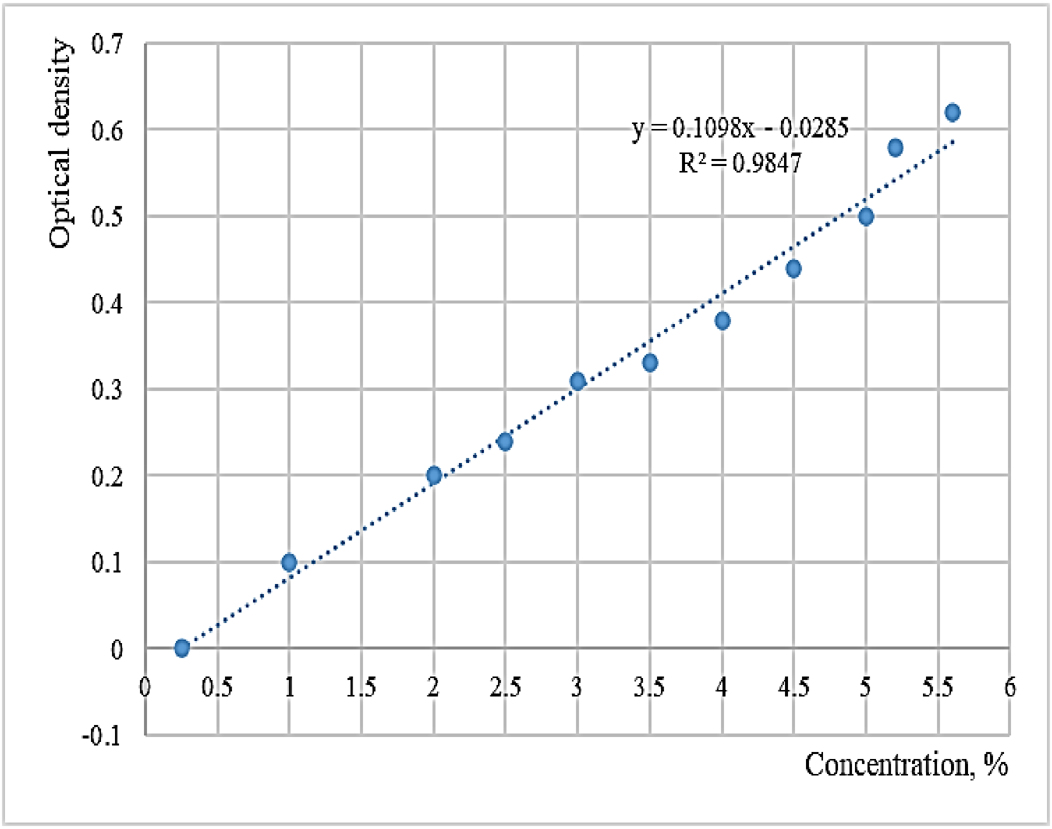

Fig. 2 Dependence of optical density of humic acid solutions from low-mineralized slit sulphide mud from their concentration.

of peloids from 0.1 to 1%, there was also a change in the total content of antioxidants by 11.7, 8.5, 9.3 times in fulvic, gimatomelanic and humic acids of peloids, respectively. From the presented results, it can be concluded that all fractions of humic substances of peloids have antioxidant properties, with little activity observed at their lower concentration. Gimatomelanic acids contain a greater amount of total antioxidants compared to fulvic and humic acids of peloids. Thus, the change in the total content of antioxidants in various fractions of humic substances increases in the series; fulvic - humic – gimatomelanic acids.

Thus, it can be stated that the effectiveness of the manifestation of the antioxidant properties of gimatomelanic, humic, and fulvic acids of peloids is linearly dose-dependent, with the most pronounced dose-dependent effect on the peloids of gimatomelanic acids. The found patterns of changes in the total content of antioxidants are probably associated with an increase in intra- and intermolecular interactions between macromolecules of humic substances with an increase in solution concentrations. The growth of interactions between functional groups of macromolecules inhibits the manifestation of humic substances and antioxidant properties. The values of the total content of natural antioxidants in humic substances of peloids make it possible to recommend them for individual therapy, as well as for the creation of new medicinal substances with antioxidant activity.

The pharmacotherapeutic effect of HS, as well as their derivatives, directly depends on the ability of compounds to penetrate biological membranes. Transportation of peloid components through biomembranes faces significant difficulties due to their high molecular weight. In addition, the structure of the HS has a stochastic type and is characterized by a high degree of disorder. In macromolecules there are active interfragmentary interactions that cause the formation of various molecular conglomerates due to ionic pairs in hydrogen bonds. It is known that HS macromolecules can be of various forms, the type of which depends on such factors as temperature, concentration of substances, pH of the medium, ionic strength and others.[20] In this regard, the problem of determining bioavailability of peloid preparations is relevant.

To obtain quantitative characteristics of dialysis, calibration graphs of the dependence of the optical density of solutions (D) from their concentrations in the linear range of 10^{-3}-$7 \cdot 10^{-3}$ (wt.) were preliminarily plotted (Fig. 2); the correlation coefficient was 0.9841.

Based on the obtained results, the degree of permeability (ε) of humic substances is calculated according to the formula (3):

$$\varepsilon = \frac{W(dialysis)}{W(initial)} * 100\% \tag{3}$$

where, $W_{(\text{dialysis})}$ is the mass fraction of HS solution after dialysis; $W_{(initial)}$ is the mass fraction of HS solutions placed in the dialyzer.

As can be seen from the data obtained (Table 3), the ability of individual HS fractions to dialysis depends on the acidity of the solutions. The highest degree of dialysis at pH 7.4 is characteristic for fulvic acids -40.1, and the lowest for humic acids -20.1. Gimatomelanic acids have an intermediate εvalue of 25.4.

Table 3. The degree of permeability of HS at different pH values of the medium.

Permeability (ε)				
pH	4.0	6.0	7.4	9.0
Fulvic Acid	7.2	39.3	40.1	41.3
Gimatomelanic Acid	5.5	23.2	25.4	30.5
Humic acid	2.7	19.7	20.1	20.9

The established regularity is in accordance with the interval of values of molar masses of fractions. The humic acid fraction, characterized by the highest molar mass value, dialyzes 1.5-2.5 times worse than the lighter fractions, which include fulvic and gimatomelanic acids. With an increase in pH to 9.0, a symbatic increase in the permeability of compounds by 8 and 9% relative to the coefficient values at pH 7.4 is observed. (Table 3). It should be noted that the change in the bioavailability of fulvic acids for dialysis with an increase in pH up to 9.0 is insignificant. With a decrease in the pH of solutions to the value of 4, there is a sharp decrease, 6– 7 times, in the degree of dialysis according to pH values given in Table 3. The above features of dialysis are determined, apparently, by the inconsistency of the shape of the conformations of humic substances. In general, the dependence of dialysis on the number of ionogenic groups in the composition of the macromolecule can be traced.The mentioned features of permeability are determined, apparently, by the inconsistency of the shape of the HS conformations. In general, there is a dependence of dialysis on the number of ionogenic groups in the macromolecule. With increasing pH values, HS macromolecules tend to adopt an elongated configuration. This is due to the increase in the degree of ionization of acid groups due to the strengthening of salt formation processes in an alkaline medium with sodium cations. At the same time, the mutual electrostatic repulsion of similarly charged fragments of the molecular structure is enhanced, which contributes to the elongation of the macromolecule and the approximation of its shape to the linear one. With decreases in pH values to 4.0, a sharp decrease in the number of molecules capable of dialysis indicates, apparently, that macromolecules take a shape close to spherical. The twisting of the macromolecule is probably due to the emergence of numerous hydrogen bonds. As a result, aliphatic and aromatic fragments of the structure prevail on the surface of the stochastic tangle, which complicates the dialysis of such conglomerates through the membranes.

The above assumptions are confirmed by the study of the permeability of fractions modified with silver ions (Table 4). The effect of cations on the dialysis of HA was carried out on the example of silver ions. The silver ion, being a soft Lewis acid, acts on macromolecules in the same directions as the sodium ion, contributing to the formation of an elongated spatial shape of macromolecules. To do this, 0.1% (w/w) solution of substances was modified by adding 0.5 ml of 0.02 mole-eq/l solution of silver nitrate.

As can be seen (Fig. 3), the dialysis of silver humate is 1.2 times more intense than the permeability of HA. Dialysis was

Table 4. Degree of dialysis of HS of peloid modified with silver ions at different pH values.

Permeability (ε)				
pH	4.0	6.0	7.4	9.0
FA-Ag	13.3	46.8	53.7	55.9
GA-Ag	7.1	32.5	43.6	47.5
HA-Ag	4.9	21.3	29.5	32.1

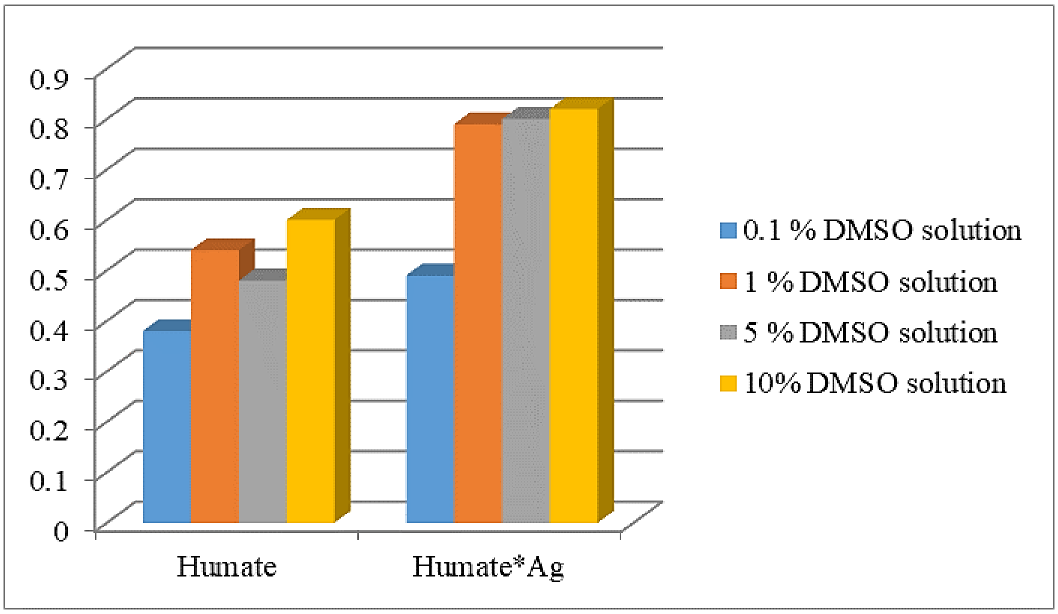

Fig. 3 Dependence of the degree of dialysis of HA and humate solutions from DMSO concentration.

carried out in the presence of the transagent dimethyl sulfoxide (DMSO) at concentrations of 1.0; 50; and 10 wt. %. As illustrated by the results of photometric measurement, DMSO increases the permeability of peloid preparations through biological membranes. 0.1% dimethyl sulfoxide solution slightly affects the permeability of HA, and when 1.0% DMSO solution is administered the permeability increases by 31.65%. For HA and silver humate, a 10% concentration of DMSO is optimal, at which a 1.6-fold increase in permeability is observed.

The obtained results indicate the fundamental possibility of HS penetration through biological membranes. Permeability depends on the acidity of the medium, the ionic strength and the presence of transagents. Bioavailability of the studied peloid preparations increases in a slightly alkaline environment and decreases in acidic, which is associated with changes in the spatial configuration of macromolecules. As a result of the experiment, we established the nature of the dependence of the penetration of HS and their complexes with silver ions through the biomembrane into the aquatic environment, depending on various factors. The slightly alkaline medium contributes to the increase of ionogenic groups with a negative charge on the surface of HS macromolecules, which, apparently, is accompanied by an increase in electrostatic repulsion of individual fragments of the molecule.

4. Conclusions

As a result of the studies, it was established that the peloid of the Tuzkol Lake deposit (Republic of Kazakhstan) are high-quality medium-sulfide, slightly alkaline slit mud. Using EPR-spectroscopy and the amperometric method of research, new information was obtained on the peculiarities of the molecular structure of humic substances from the low-mineralized slit sulphide mud of the Tuzkol deposit. It was found that the high content of PMC in HS characterizes the presence of free radicals in them, the concentration of which is $3.75 \cdot 10^{18}$ spins/g, which determines the prospects of using HA as antioxidant natural remedies. Analysis of the concentration of paramagnetic centers shows that the minimum number of free radicals contains molecules of fulvic acids, and the maximum number of free radicals contains molecules of humic acids of peloids. The results of the amperometric determination of antioxidant properties of HS of the studied low-mineralized silt sulphide mud deposits of Tuzkol indicate that they have antioxidant activity, which allows them to be used as a natural biologically active substance for medical products. It has been shown that the change in the total content of antioxidants in various fractions of humic substances increases in the series; fulvic - humic – gimatomelanic acids. Bioavailability of the studied peloid preparations increases in a slightly alkaline environment and decreases in acidic, which is associated with changes in the spatial configuration of macromolecules. As a result of the experiment, we established the nature of the dependence of the penetration of HS and their complexes with silver ions through the biomembrane into the aquatic environment, depending on various factors.

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Conflict of Interest

There is no conflict of interest.

Supporting Information

Not applicable.

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