

# Features of Obtaining and Properties of Fulvic Acid from the Peat of Nizhny Novgorod Region

Konnova, M. A<sup>1\*</sup>, Volkov, A. A<sup>1</sup>, Kostryukov, S. G<sup>2</sup>, Melnikova, N. B<sup>3</sup>

<sup>1</sup>Department of Pharmaceutical Chemistry, Privolzhsky Research Medical University, 10/1 Minin Sq., 603950 Nizhny Novgorod, Russia

<sup>2</sup>Department of Organic Chemistry, National Research Ogarev Mordovia State University, 68 Bolshevistskaya str., 430005 Saransk, Russia

<sup>3</sup>Faculty of Chemistry, Lobachevsky State University, 23 Gagarin Av., 603022 Nizhny Novgorod, Russia

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\*Corresponding author: Konnova, M. A

Department of Pharmaceutical Chemistry, Privolzhsky Research Medical University, 10/1 Minin Sq., 603950 Nizhny Novgorod, Russia

## Abstract

Properties of fulvic acid (FA) extracted from the peat of the Nizhny Novgorod region of Russia by ultrasonic extraction with alkaline or ethanol solutions and further purification were studied in the paper. The purification of FA by the Lamar method was shown to possibly lead to the formation of FA polymorphs of the same composition but different structure (IR, solid-state <sup>13</sup>C NMR, fluorescence, UV, energy-dispersive X-ray spectroscopy, SEM and optical microscopy, concentration of carboxyl and phenol groups). Aggregation of FA polymorphs in water led to the formation of nanoparticles with the average size of 8–10 nm and the zeta potential of –22 to –27 mV. The formation and properties of polymorphic supramolecular structures are greatly influenced by the drying mode. The solubility of the two FA polymorphs varied from freely soluble (3.3 mL of water per gram) after the FA solution freeze-drying to soluble (12 mL of water per gram) after the FA solution vacuum drying. The FA sample extracted by ethanol ultrasonic extraction from the peat, followed by convection drying, had a solubility of 666.7 mL of water per gram (Slightly soluble), a zeta potential of -6 mV, and a particle size of 15–20 nm.

**Keywords:** Fulvic acid, peat, fulvic acid solubility, alkaline and alcohol extraction, drying, Lamar method, ultrasound treatment.

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## INTRODUCTION

Humic (HA) and fulvic (FA) acids being the components of natural sources such as silt, soil, peat, lignin, coal, and others, are promising biologically active substances for pharmacy and medicine. This group of supramolecular or polymeric compounds of various composition and structure exhibits powerful antioxidant [1-7], antitumor [8-11], hypoglycemic [12-14], antiviral [15-18], and anti-inflammatory properties [3, 15, 19-22].

The important factor for the manifestation of these biological effects is the antioxidant activity of these compounds. Antioxidant properties are largely due to the phenolic nature of this group of compounds. In the structure of both HA and FA, phenolic fragments, including catechol ones, also contain other groups (for example, carbonyl, carboxyl, methoxy). Due to this

structure, phenolic compounds are capable of stepwise electron donation and exhibit antioxidant activity by two mechanisms: 1) ROS inactivation; 2) chelation of metal ions of variable valence [23-26], and also normalize the level of antioxidant defense enzymes. The reduced form has more antioxidant properties than the oxidized quinoid form.

The advantage of this group of compounds is their wide distribution in nature, these compounds are obtained from renewable raw materials. On the other hand, depending on the raw material, not only the structure of FA and HA changes, but also their ratios in the raw material. Usually, HA and FA differ in two main criteria: 1) molecular weight is greater than 1 kDa for HA and less than 1 kDa for FA [27, 28]; 2) solubility in water and others media with different pH: FA is highly soluble in water and in an acidic medium,

in contrast to HA, which is readily soluble in an alkaline medium [29-36].

The physicochemical and biological properties of FA depend not only on the natural source, but also on the method of isolation, concentration, purification, drying at all the stages of the process. The most traditional method for extracting FA is their primary extraction with alkaline solutions (NaOH, KOH, NH<sub>4</sub>OH), organic solvents (ethanol, dimethyl sulfoxide), and water [37-46]. The process is usually intensified via physical radiation or ultrasonic treatment [47-50]. The secondary step is usually the separation of FA from HA using acid treatment. The concentration of FA and its purification is usually carried out using the Lamar method, based on selective sorption by the nonionic polymer resin at low pH [51].

In general, it should be noted that depending on the feedstock, its place of origin, and FA synthesis, all major extraction methods require modification to obtain the product with the desired biological activity and physicochemical properties. In an alkaline medium and/or during drying the oxidation of the product at phenolic hydroxyl to quinoid structures and other chemical transformations are possible. Therefore, in order to use FA as an active pharmaceutical ingredient (API) with the desired physicochemical properties, controlled size and solubility, it is important to adapt the process for obtaining FA at all stages for a specific raw material.

In this paper, we studied the improvement of the FA extraction from the peat in the Nizhny Novgorod region (Russia) due to ultrasonic treatment during alkaline and alcohol extraction, the use of the resins with a higher sorption capacity for selective FA sorption, and also investigated the effect of the FA drying conditions on the structure and solubility.

## MATERIALS AND METHODS

### Materials

Lowland Peat of Nizhny Novgorod region (Russia) was received from LLC "ESSON" (Nizhny Novgorod, Russia).

In study we used sodium hydroxide (99.0% purity, Bashkir Soda Company JSC, Sterlitamak, Russia), sulfuric acid (99.9% purity, EKOS-1, Staraya Kupavna, Russia), ethanol (95.0% purity, Vekos, Nizhny Novgorod, Russia), Superlite™ DAX-8 (Supelco - Sigma-Aldrich Co, Bellefonte, PA, U.S.A.), Cationite KU 2-8 H<sup>+</sup> (NevaReactiv, Saint Petersburg, Russia)

### Fulvic acid preparation

- 1) 1000 mL of 0.1 M NaOH was added to the peat sample (200 g), the mixture was heated at 80°C and sonicated for an hour using ultrasound bath (Sapphire - 1.3 1/2 TTC,

35kHz, LLC "NOVOLAB", Novosibirsk, Russia). The resulting alkaline solution was centrifuged at 3000 rpm for 15 min until precipitation of humin. Humic acid was precipitated after the dropwise addition of H<sub>2</sub>SO<sub>4</sub> conc. to pH 1-2 and then the dispersion was centrifuged again to fully remove HA and separate FA solution. The FA solution was passed through the top of the column filled with Supelite DAX-8 resin using a peristaltic pump. After passing the fulvic acid solution through the column, the column was washed with distilled water until the effluent became colorless. Desorption of fulvic acid was carried out by back elution with 2000 mL of 0.1 M sodium hydroxide solution to obtain a strongly alkaline solution (pH near 10) of sodium fulvate (Na-FA). Then the Na-FA solution was passed through the column filled Cationite KU 2-8 H<sup>+</sup> resin for the Na-FA protonation. Drying of the FA solution was carried out by two methods: freeze-drying (-80 to -40°C for 8 h – LGJ-10, Vikumer, Beijing, China) and vacuum drying (1 mmHg, 50°C for 6 h – Smart Evaporator K4, BioChromato, Fufisawa, Japan).

- 2) 1000 mL of 95% ethanol was added to the peat sample (200 g). The mixture was thoroughly mixed and sonicated using ultrasound bath (Sapphire - 1.3 1/2 TTC, 35kHz, LLC "NOVOLAB", Novosibirsk, Russia) for 30 minutes. The resulting extract was centrifuged at 3000 rpm for 15 min until complete precipitation of humin and humic acid. The alcoholic extract was dried up to constant weight (100±5°C – ShS-10-02 SPU, JSC "Smolensk SKTB SPU", Smolensk, Russia).

Total nitrogen was assayed by Kjeldahl method (VELP Kjeldahl Systems, VELP Scientifica, Usmate (MB), Italy).

### Solid-state <sup>13</sup>C NMR

Registration of solid-state <sup>13</sup>C NMR spectra was performed by JNM-ECX400 spectrometer (JEOL, Tokyo, Japan – 9.39 T, 100.5 MHz) in the solid phase at room temperature using the technique of cross-polarization and magic angle rotation (combined CP-MAS experiment) with frequency 10 kHz rotation in 4 mm zirconia rotors. The magic angle of sample rotation (MAS) was determined at a rotation speed of 6 kHz using a KBr standard sample. The CW (Continuous Wave) decoupling method was used. VACP variable amplitude cross-polarization, RAMP-CP method were used. Adamantane (29.5 ppm) was used as a reference sample; the resolution was also adjusted using it. All CP-MAS experiments were performed at room temperature; proton decoupling was performed using double phase pulse modulation (TPPM). The duration of the 90° pulse for <sup>13</sup>C nuclei was 2.93 μs and was

determined using a sample of hexamethylbenzene. The total number of scans was 4096.

The spectra were recorded using the Delta 4.3.6 program and processed using the ACD/NMR Processor Academic Edition, Ver. 12.01.

#### FTIR Analysis

FTIR spectra were obtained in the 400–4000  $\text{cm}^{-1}$  range by an IR Prestige-21 FTIR spectrometer (Shimadzu, Kyoto, Japan). The resolution was 0.5  $\text{cm}^{-1}$ , and the number of scans was 45.

#### UV analysis

UV spectra were recorded by UV-1800 (Shimadzu, Kyoto, Japan).

#### Photoluminescence analysis

Fluorescent spectra were obtained using spectrofluorimeter CM 2203 (Solar, Minsk, Republic of Belarus). One-dimensional emission spectra were taken in the wavelength range of 400–550 nm at a constant excitation wavelength of 360 nm. Excitation spectra were recorded in the range of 300–500 nm at a fixed radiation wavelength of 520 nm.

#### SEM and EDXMA Studies

The samples were visualized by scanning electron microscopy (SEM) using a JSMIT300LV (JEOL, Tokyo, Japan) microscope with an electron beam diameter of about 5 nm and a probe current below 0.5 nA (operating voltage 20 kV). The surface topography of the powders was studied using low-energy secondary electrons and backscattered electrons. The elemental composition of the powders was studied using X-ray microprobe analysis (XRM) with an X-MaxN 20 detector (Oxford Instruments, Oxfordshire, England).

#### Direct Potentiometric titration of FA

The analysis was performed in accordance with the methodology [52]. 100 mL of the FA solutions (10 mg/%) were titrated by 0.1M NaOH and 0.1M  $\text{Na}_2\text{CO}_3$ . The system was stirred until equilibrium was established after each addition of titrant (0.2 mL). The equivalent points were determined from values of pH at the end of the titration of carboxyl (pH 7.0) and hydroxyl (pH 10-11) groups.

#### Back potentiometric titration of FA

The analysis was performed in accordance with the methodology [52]. 0.1M NaOH solution was added to the FA solution up to the values pH 12. The titrant was 0.1M HCl. The system was stirring until the stable pH value was obtained.

#### Determination of acid groups via acetate method

The analysis was performed in accordance with the methodology [52]. The FA (0.01 g) was treated with 2 mL of ethanol; 10 mL of a 0.1M solution of sodium acetate and 40 mL of water were added. The reaction mixtures were stirred at 100°C in a flask with a reflux condenser for 40 min. The precipitate was filtered off and thoroughly washed with 100 mL of water and the released acetic acid was titrated by 0.05M KOH in the presence of phenolphthalein. The content of acid groups  $C^S$  was calculated by the equation:

$$C^S = \frac{V(\text{KOH}) \cdot C(\text{KOH}) \cdot V_1}{m \cdot V_2} \text{ (mmol-eq/g)},$$

Where  $V(\text{KOH})$  is the volume of 0.05M KOH used for the titration of the sample, mL,  $C(\text{KOH})$  is the concentration of the KOH solution, m is the weight of the FA sample,  $V_1$  is the total volume, mL;  $V_2$  is the volume of the aliquot, mL.

#### The particle size analysis

Particle size distribution in the solutions was determined with dynamic light scattering using Nanowin particle size analyzer (LLC “Microtrak”, Saint Petersburg, Russia).

#### Zeta potential analysis

Zeta potential was measured by Zetachek particle charge reader (LLC “Microtrak”, Saint Petersburg, Russia).

#### pH analysis

The pH of the solutions was measured by “Expert-pH” pH meter (LLC “Basis Group”, Saint Petersburg, Russia).

## RESULTS AND DISCUSSION

The figure 1 shows general schemes of fulvic acid preparation used in the paper. According to the first method, FA was extracted from the peat by ultrasonic extraction with 0.1 M NaOH followed by HA precipitation and pH adjustment to 2 with concentrated  $\text{H}_2\text{SO}_4$ . Humic and humic acids were removed by centrifugation. The final light brown FA solution was purified using the Lamar method as described in the experimental section. In our work, the sodium salt of fulvic acid was converted into fulvic acid by protonation using the strongly acidic cationite KU 2-8  $\text{H}^+$ , which makes it possible to increase the efficiency of FA extraction from 65% (using Amberlite IR120  $\text{H}^+$ ) to 73%. The FA concentration was monitored by fluorimetric and UV assay. For comparison, FA was extracted by ultrasonic extraction from the ethanol solution with multiple stepped centrifugation at room temperature to remove humin and humic acids.

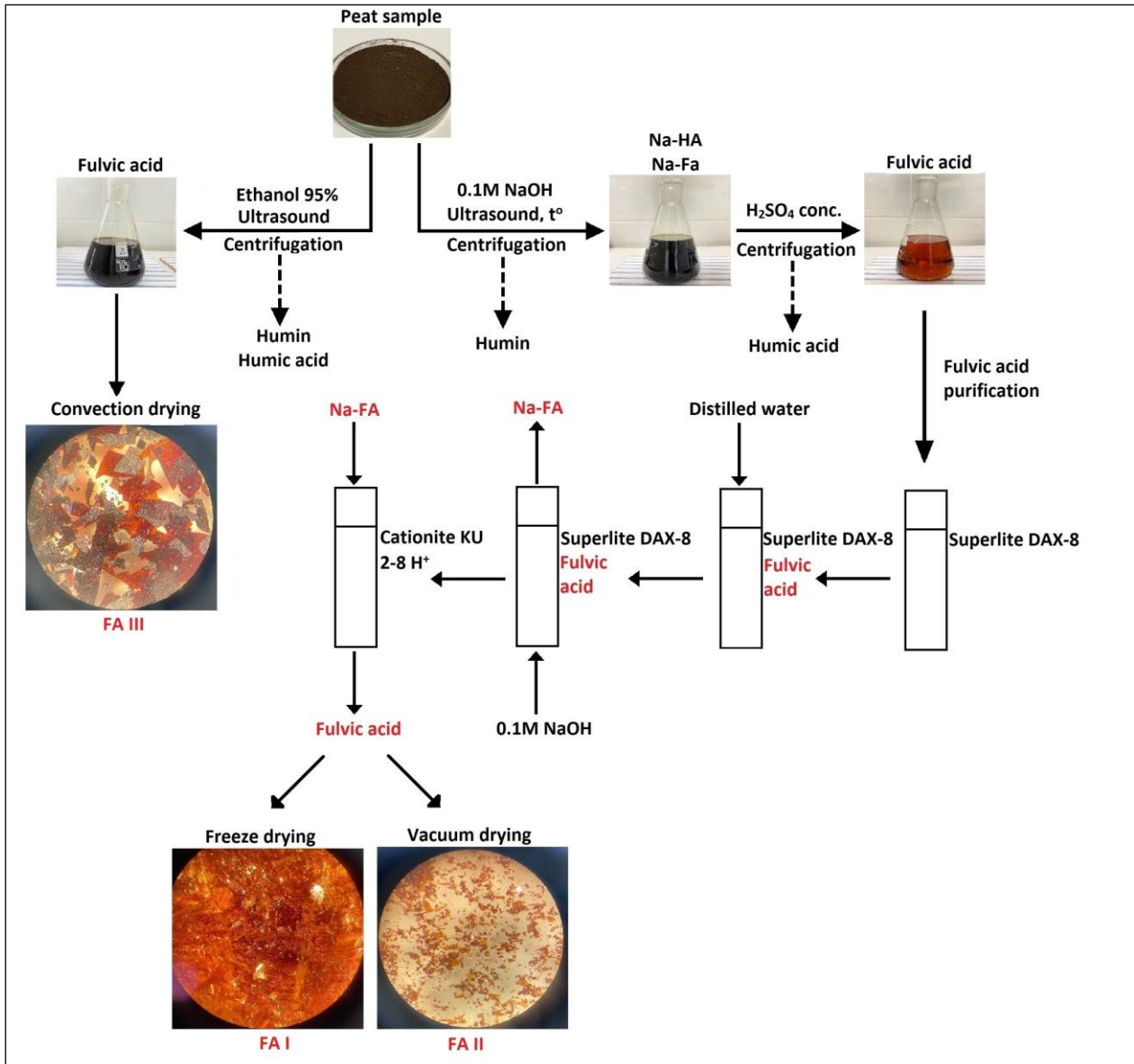


Figure 1: The methods of the FA preparation from the peat used in the work

The resulting FA samples had the different appearance (color, shape of crystals) depending on the method of isolation and drying of FA. Figure 2 shows photographs of typical samples obtained by the combination of: 1) ultrasound-assisted alkaline extraction of the peat with the Lamar method FA

obtaining and following freeze-drying (FA I); 2) ultrasound-assisted alkaline extraction of peat with the Lamar method FA obtaining and following vacuum drying (FA II); 3) ultrasound-assisted alcohol extraction of peat and following convectional drying at 100 C (FA III).

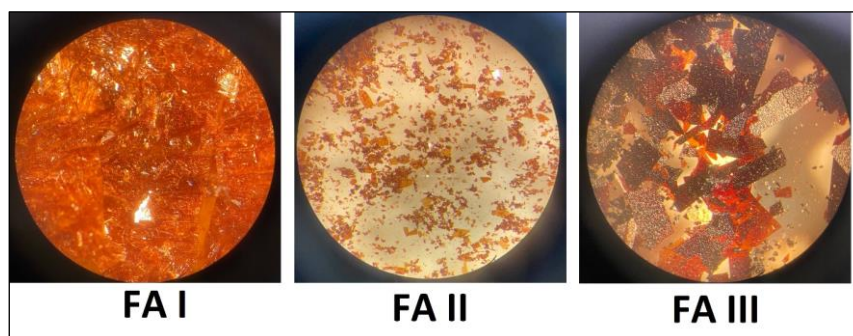


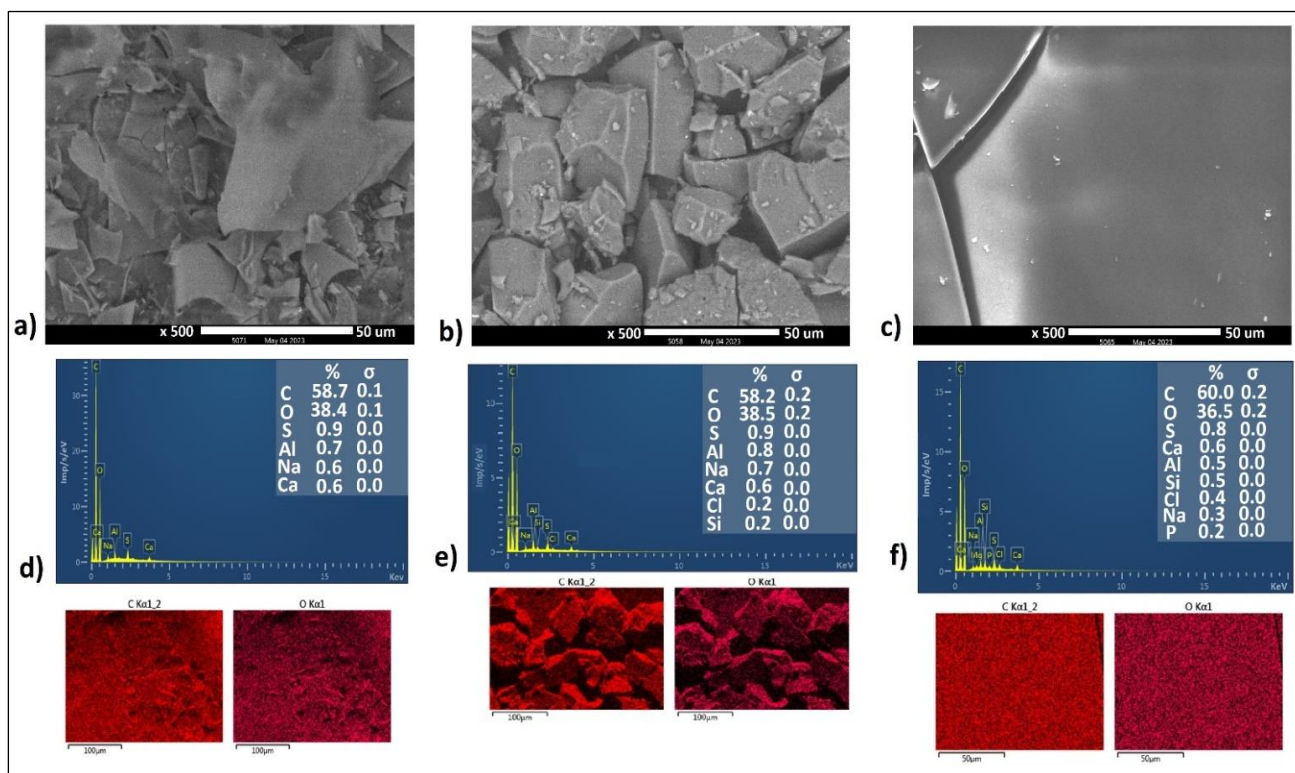
Figure 2: Photos of the different FA samples at twice magnification

Freeze drying of the fulvic acid solution led to the production of the gold-orange FA I as air needle plates. Drying the same FA solution under vacuum resulted in the yellow-brown FA II in the form of transparent plates. Convection drying of the alcoholic extract led to the production of the dark brown FA III as plates.

Figure 3 shows typical SEM images of the FA and EDX spectra. The SEM images also show the different structure of the FA samples at higher

magnification. The EDX spectra characterized the FA I, II, III powders as highly dispersed with a uniform distribution of elements throughout the volume, with the C:O ratio approximately equal to 1.5 (Table 1).

Analysis of all three FA samples showed that the obtained samples, while having the same elemental composition, differed in solubility. The solubility of FA I is 200 times greater than FA III and 4 times greater than FA II.



**Figure 3:** SEM images (a–c) and EDX spectra and the distribution of elements in powders on aluminum foil (d–f) for FA I, FA II, FA III, respectively

In the Table 1 the characteristics of three structural modifications are shown.

**Table 1: Characteristics of the three FA forms**

Properties	FA I	FA II	FA III
Appearance	light brown air spikes	light brown transparent plates	dark brown sintered plates
Solubility	<b>3.3 mL of water per gram (Freely soluble)</b>	<b>12.5 mL of water per gram (Soluble)</b>	<b>666.7 mL of water per gram (Slightly soluble)</b>
C, %	56.0±2.1	56.0±2.2	59.0±1.2
O, %	38.0±2.3	38.0±2.1	36±2.0
Acid carboxyl groups, mmol-eq/g	7.1±0.03	6.8±0.01	6.2±0.04
Phenol groups, mmol-eq/g	4.8±0.01	4.6±0.02	4.0±0.03
Total acid groups, mmol-eq/g	12.0±0.05	11.4±0.03	10.2±0.02
Zeta-potential, mV	-27.9±0.21	-25.6±0.22	-6.4±0.25
pH (0.01%)	2.1±0.15	3.0±0.10	5.1±0.20
D <sub>av.</sub> , nm (DLS)	8.0±0.65	10.0±0.51	20.0±0.51

Assay of the acid groups, both carboxyl and phenolic, in the FA samples (FA I, FA II, FA III) showed their high content: from 6.2 to 7.1 for carboxyl

groups and from 4.0 to 4.8 for phenolic groups respectively (Table 1). The ratio of carboxyl groups to

phenolic groups in the FA samples is approximately 1.5 or 4:2 in the FA.

The data of FTIR spectra confirm the presence of these groups in the FA. (Figure 4, Table 2).

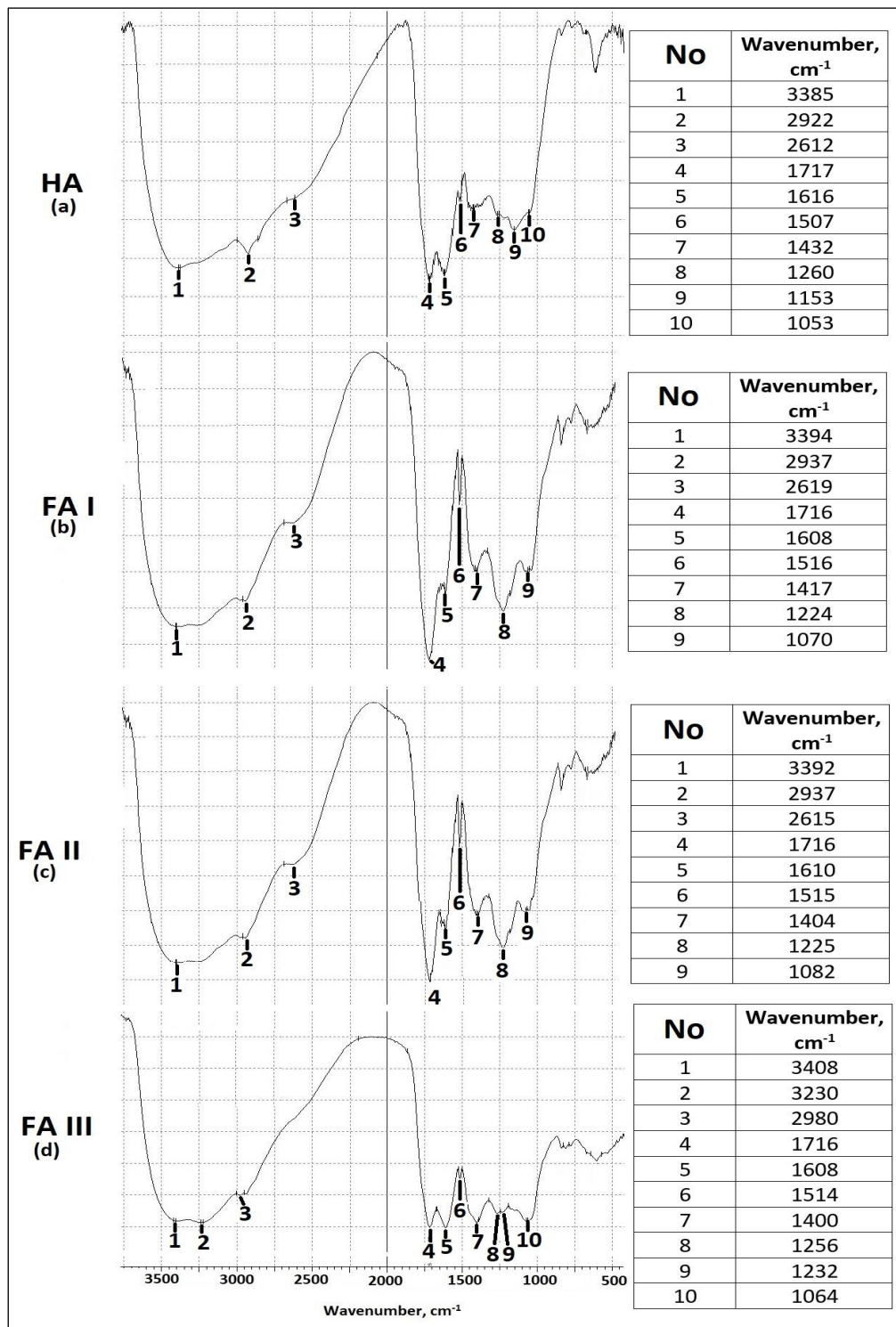


Figure 4: FTIR spectra (a-d) of the FA and HA

Wide absorption band in the region of 3600-2500 cm<sup>-1</sup> with a maximum in the range of 3400-3200 cm<sup>-1</sup>. correspond to stretching vibrations of hydroxyl groups -O-H (in the composition of phenols, alcohol and carboxyl groups). Also, in the region above 3000

cm<sup>-1</sup> there are bands of stretching vibrations =CH bonds of the aromatic ring. The presence of the 1716-1717cm<sup>-1</sup> band is due to the stretching vibrations of the C=O carbonyl groups. This group can be represented mainly by aldehydes, ketones, carboxylic acids and their

derivatives. The band at 2750-2500  $\text{cm}^{-1}$  indicates a possible dimerization of the acid groups. The carboxylate ion gives bands of antisymmetric stretching vibrations in the region of 1650-1550  $\text{cm}^{-1}$ , and a weaker band corresponding to symmetric stretching vibrations around 1400  $\text{cm}^{-1}$ . Also, in the region of 1400  $\text{cm}^{-1}$  there are bending vibrations of phenolic groups O-H. Strong absorption near 1720  $\text{cm}^{-1}$  and also

at 1200  $\text{cm}^{-1}$  indicate that fulvic acid contains a high amount of carboxyl and carbonyl groups. The possible presence of esters and lactones is indicated by antisymmetric stretching vibrations 1280-1150  $\text{cm}^{-1}$ . We used the ratio of the intensity of stretching vibrations (T) of the carboxyl groups as the reference band to the carbonyl, phenolic and alcohol groups (Table 2) to characterize the structure of the FA.

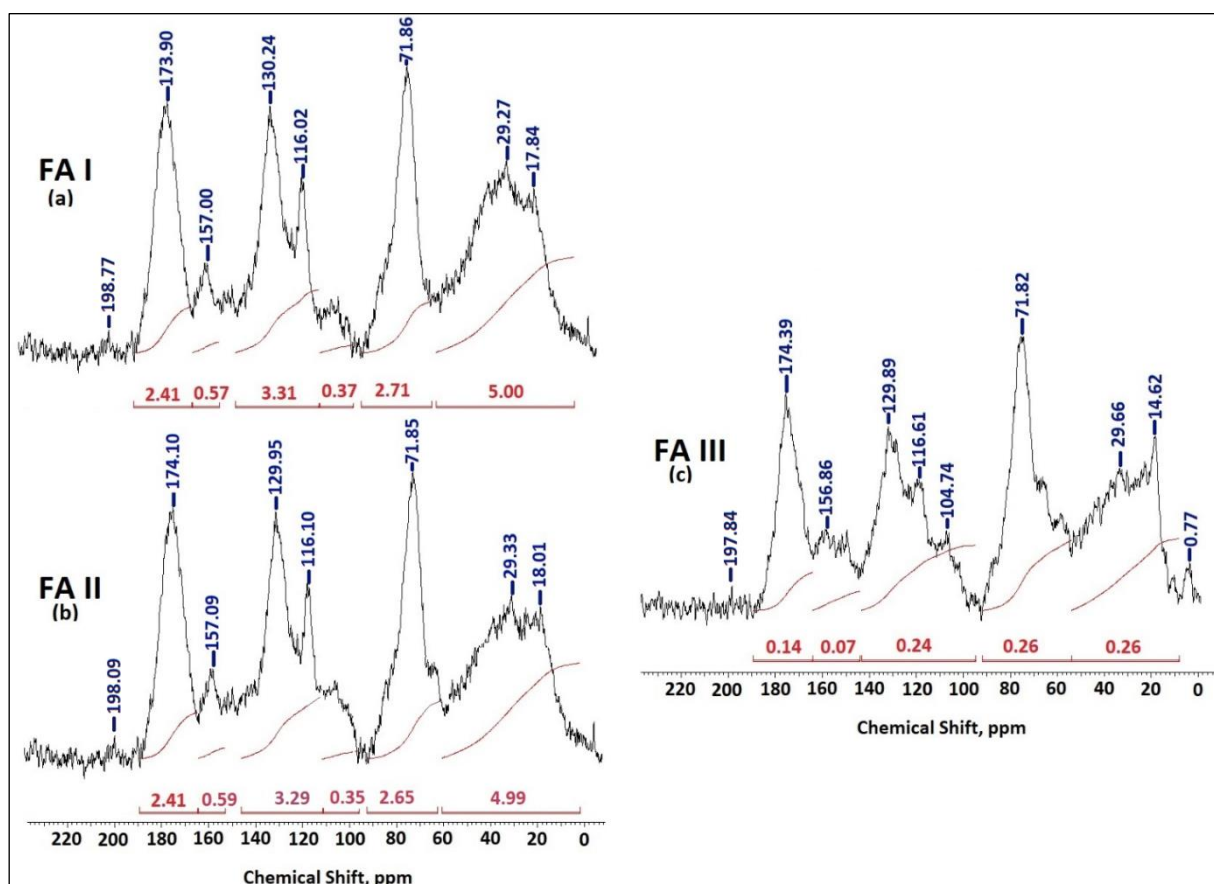
**Table 2: Ratios of absorption bands of humic derivatives**

Ratio of absorption bands	$T_{\text{COOH}}/T_{\text{C=O}}$	$T_{\text{COOH}}/T_{\text{Ph-OH}}$	$T_{\text{COOH}}/T_{\text{OH in alcohol}}$
	$T_{1600-1650}/T_{1716-1720}$	$T_{1600-1650}/T_{1200-1270}$	$T_{1600-1650}/T_{1020-1075}$
FA I	2.16	1.17	0.84
FA II	2.10	1.15	0.87
FA III	0.99	0.66	0.81
HA	1.05	0.69	0.68

**Note:** HA was obtained after ultrasound-assisted alkaline extraction and precipitation by  $\text{H}_2\text{SO}_4$

The structure of the obtained products was proved by solid-state  $^{13}\text{C}$  NMR spectra (Figure 5). The spectra contain distinct signals related to aliphatic (0-50 ppm), aromatic (108-165 ppm), and carboxyl (165-190 ppm) carbon atoms. In addition, there are signals in the region of 60-96 ppm, which correspond to O-substituted carbon atoms, for example, alcohol or ether fragments. In the aromatic part of the spectrum, there are signals of O-substituted carbon atoms 160-140 ppm.

and more intense signals in the region of 120-140 ppm, in addition, there are signals of 115-120 ppm belonging to olefinic carbon atoms associated with the CO group. Low-intensity signal 95-106 ppm refers to hemiacetal carbon. The presence of carboxyl confirms an intense signal at 170-180 ppm, and the presence of a carbonyl group confirms a low-intensity signal in the region of 197-198 ppm.



**Figure 5: Solid-state  $^{13}\text{C}$  NMR spectra (a-c) of the FA samples**

The NMR spectra were analyzed by the ratio of signal intensities of alcohol groups to carboxyl groups, phenolic groups, and pyranone ring. The reference band is the signal corresponding to O-substituted carbon atoms in alcohol fragments. Table 3

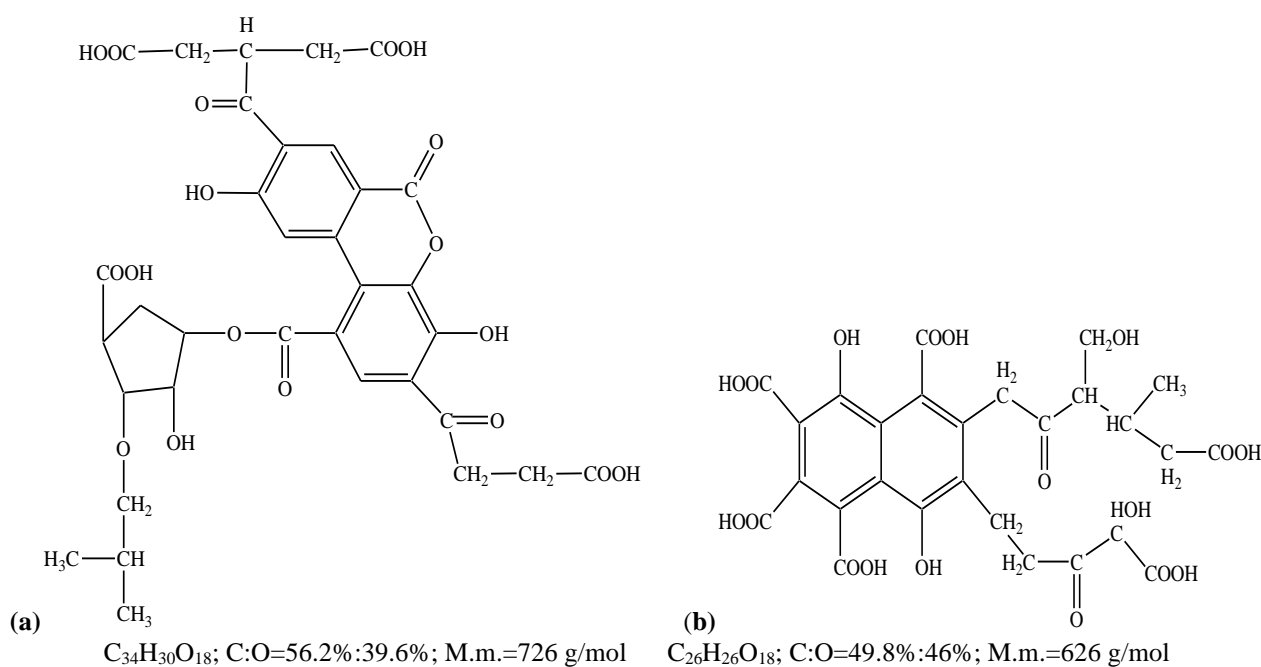
shows the ratios of the signals in the spectra. From the presented data, it can be seen that the ratio of alcohol and carboxyl groups is close to 1. And the ratio of alcohol to phenolic groups is close to 1.5.

**Table 3: Ratios of the signals intensity (I) of the NMR spectra of the FA samples**

Signal ratio	I <sub>60-80</sub> /I <sub>160-180</sub>	I <sub>60-80</sub> /I <sub>120-140</sub>	I <sub>60-80</sub> /I <sub>100-120</sub>	I <sub>60-80</sub> /I <sub>10-40</sub>
FA I	1.13	1.14	1.56	1.49
FA II	1.10	1.12	1.58	1.50
FA III	1.28	1.54	2.27	2.13

Based on solid-state <sup>13</sup>C NMR, FTIR, ratio of carboxyl and phenol groups (4:2), ratio of carbon to oxygen (1.5) and solubility, it can be assumed that the FA samples we obtained have fragments similar to structures shown in Figure 6. Determination of the

molecular weight of FA in the FA I sample by cryoscopy showed the M.m.=740 g/mol (solvent - water). The nitrogen content in the FA I sample was equal to 0.17%.

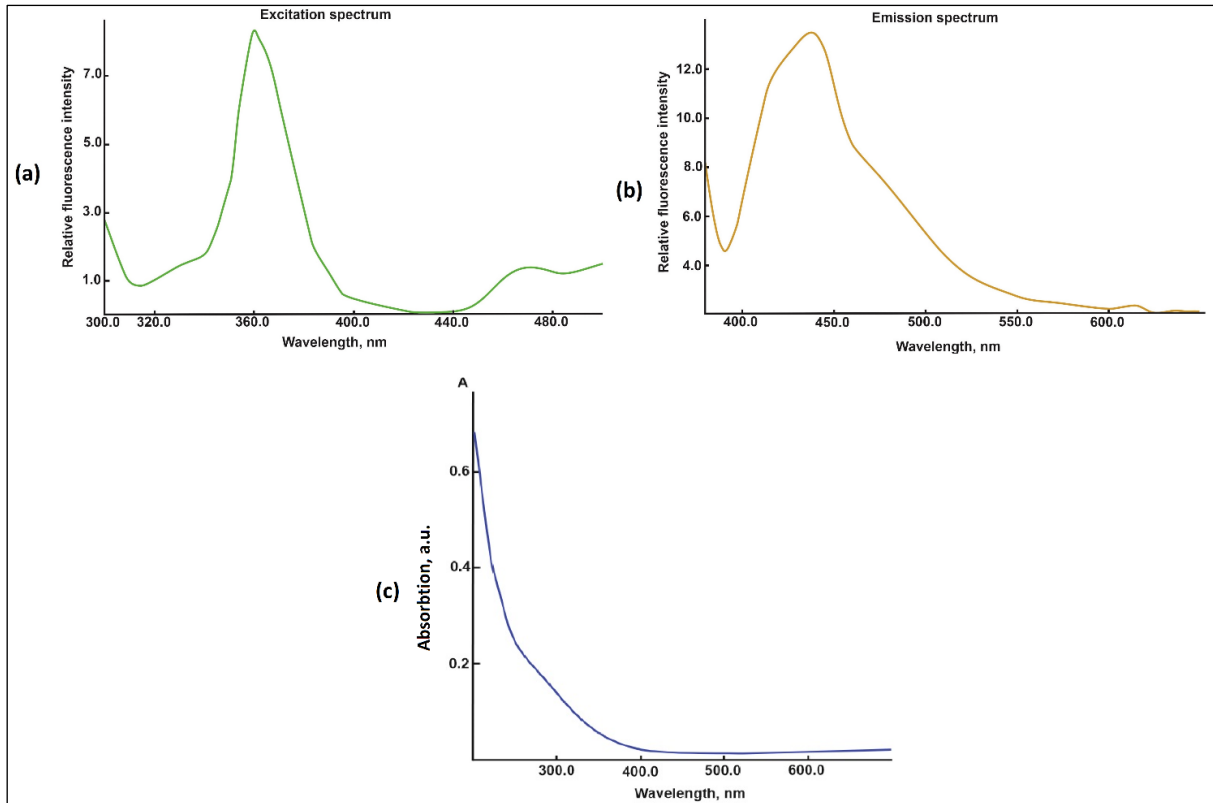


**Figure 6: Supposed general formulas of fulvic acid (a, b)**

The structural packing of the FA crystals depends on intra- and intermolecular hydrogen bonding, which can promote the formation of various supramolecular structures. In presented experiment the process depended on the drying conditions. In the solid state, the obtained FA can crystallize into various polymorphs, - FA I and FA II, differing not only in crystal structure, but also in solubility. In the process of dissolution FA behave like polyelectrolytes, dissociating with the generation of a proton, while pH value changes depending on the concentration of FA in the solution [53]. FA molecules forming supramolecular structures are able to aggregate into

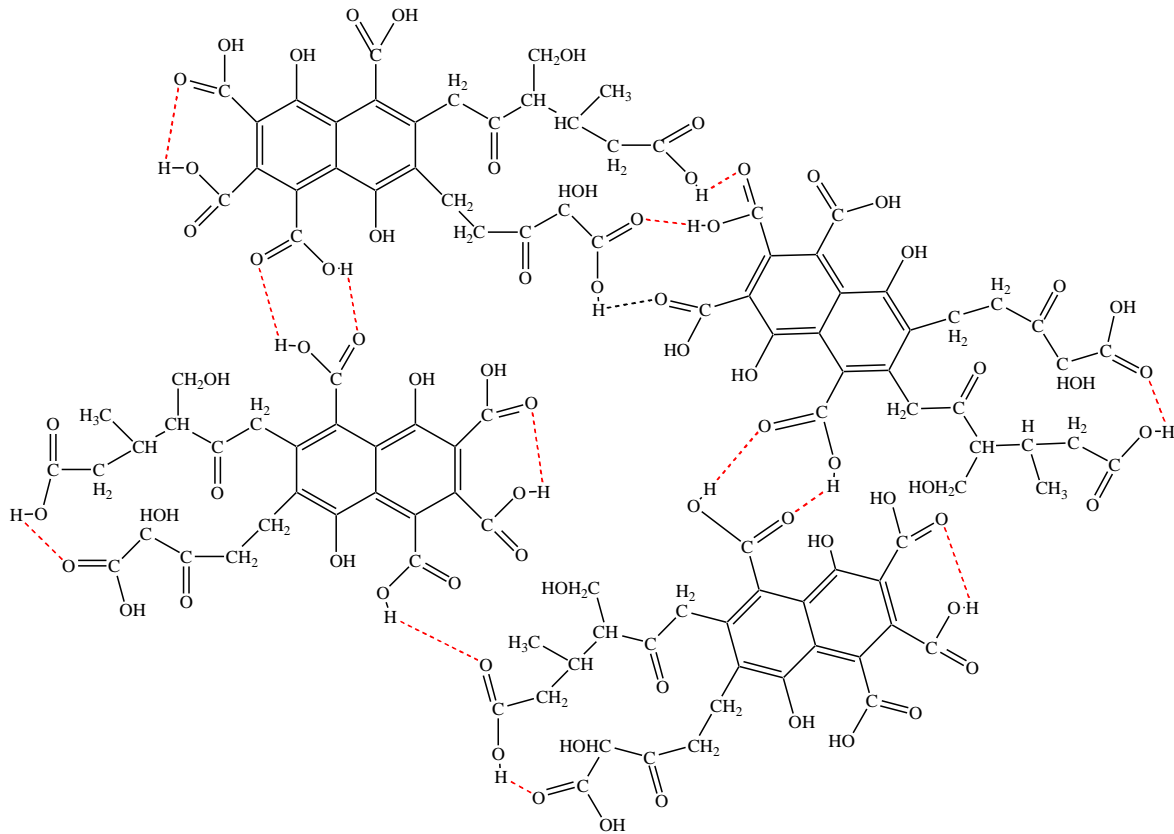
nanoparticles, the average size of which varies from 8–10 nm (FA I, FA II) to 20 nm (FA III). The resulting colloidal particles are characterized by a negative zeta potential (-25 – -6 mV), which increased with decreasing particle size (Table 1). The nanoscale nature of FA dispersions is reflected in fluorescence spectra close to literature data for FA obtained from other sources [54-57] (Figure 7). The emission spectrum of the FA I and FA II solution had the typical wide band at 445 nm with the shoulder at 470 nm and at 420 nm. The excitation spectrum had the band at 350 nm and the weak band about 440 nm.





**Figure 7: Fluorescence spectra (a, b) and UV-spectrum (c) of the 0.05% FA aqueous solution**

The scheme illustrating intra- and intermolecular binding in FA with the structure from Figure 6(b) is shown in Figure 8.



**Figure 8: Illustration of intra- and intermolecular bonding in FA**

It was shown that the drying mode had the strongest effect on the structure of the FA I and FA II polymorphs. Freeze drying reduced the formation of higher molecular weight structures [58, 59] compared to vacuum and convection drying. With low temperature stepwise lyophilization, sticking and aggregation of fulvic acid molecules is minimal. Vacuum and convection drying cause more intense degradation and structural changes associated with higher temperatures and drying rates. In addition, during freeze drying an effective reduction in the oxidation of phenolic compounds is observed.

The integration of ultrasonic treatment into the FA extraction process made it possible to significantly increase the efficiency of extracting FA from the used peat. This effect is due to the ability of the reagent (alkali or ethanol) to penetrate deep into the material, providing more intensive mixing and better distribution of the solvent inside the matrix. This leads to improved mass transfer between the phases, which in turn results in a more complete release of FA.

The increase in the efficiency of the FA extraction process by the Lamar method also occurred due to the selectivity of the FA extraction using Cationite KU-2-8 H<sup>+</sup>, which has a higher cationic capacity.

## CONCLUSION

In this paper, two main methods for extracting FA from the peat in the Nizhny Novgorod region of Russia are optimized: 1) ultrasonic extraction with ethanol; 2) the combination of ultrasonic alkaline hydrolysis followed by selective sorption of FA. The use of ultrasonic treatment and the use of the more efficient sorbent make it possible to intensify the extraction process, reduce the time and increase the yield of FA by 8%. The choice of drying method plays a key role in maintaining the quality and structural integrity of fulvic acid. Freeze drying, due to its characteristics, represents the preferred option for minimizing the oxidation of phenolic compounds and reducing the formation of higher molecular weight structures, which is important for the long-term stability and functionality of this compound. We have shown that, depending on the drying mode of the same solution, it is possible to obtain polymorphic forms with different solubility (from 3.3 mL to 12.5 mL of water per gram), but the same composition, confirmed by data on the number of carboxyl and phenol groups, elemental analysis, FTIR, and solid-state <sup>13</sup>C NMR spectra.

The high concentration of acidic groups (11-12 mmol-eq/g), the easy solubility of the FA I polymorph, and the high efficiency of its production from the peat of the Nizhny Novgorod region of Russia allow us to propose FA I as a potential API for pharmacy and medicine.

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