Radiochim. Acta **98**, 1–6 (2010) / **DOI** 10.1524/ract.2010.1695

A new technique for tritium labeling of humic substances

By G. A. Badun[1,*], M. G. Chernysheva[1], Z. A. Tyasto[1], N. A. Kulikova[2], A. V. Kudryavtsev[3] and I. V. Perminova[3]

[1] Radiochemistry Division, Chemistry Department, Lomonosov Moscow State University, 119991 Moscow, Russia
[2] Department of Soils, Lomonosov Moscow State University, Moscow, Russia
[3] Organic Chemistry Division, Chemistry Department, Lomonosov Moscow State University, Moscow, Russia

(Received June 8, 2009; accepted in revised form September 14, 2009)

Humic substances / Tritium labeling / Tritium thermal activation method

Summary. Humic substances (HS) of different origins have been labeled with tritium by the thermal activation method. Specific radioactivity of labeled HS (^{3}H-HS) was sufficiently high and varied from 0.05 to 0.6 TBq/g. Parent HS and ^{3}H-HS were analyzed by size exclusion chromatography with radioactivity and UV detection. The results allowed concluding that (1) neither partial decomposition nor polymerization of HS occurred during labeling and (2) tritium labeled molecules have a regular distribution among HS fractions of different molecular weights. The performed correlation analysis revealed that there was no significant relationship between HS properties and specific radioactivity of the obtained ^{3}H-HS. Thus universality of the developed technique for radioactive labeling of HS with tritium could be demonstrated.

1. Introduction

Humic substances (HS) are a chemically heterogeneous class of polymeric organic compounds that are widespread in aquatic and terrestrial environments. HS cannot be described by unique, chemically defined molecular structures. They are operationally defined by a model structure constructed on the basis of available compositional, structural, functional, and behavioral data and containing the same basic structural units and the same types of reactive functional groups [1]. HS play an important role in the environment due to influencing transport and fate of toxicants and nutrients and possessing physiological activity in relation to biota. In spite of the fact that HS have been the subject of numerous scientific studies, quantitative determination of HS in both environmental samples and organisms is still an unsolved problem. The radioactive labeling is a good method for studying properties of HS and their behavior in different systems including environmental one [2]. A choice of the most convenient radioisotope and labeling method is still questionable. Radiolabeled HS are required to be identical to the initial material. Thus, only a limited number of elements is preferred for labeling. On the other hand, the radiolabeled sample must keep its properties over a long period of time.

Methods of radiolabeling of HS can be divided in three groups: (1) addition of a labeled precursor to a soil sample during composting; (2) synthesis of model polymeric compounds under defined conditions; (3) direct labeling of humic materials. The two first techniques were used to introduce ^{14}C-label in HS [3–6]. Polymeric compounds used as a model for the description of HS are synthesized either by enzyme mediated (usually initiated by adding H_2O_2 in the presence of horseradish peroxidase) oxidative polymerization of phenolic compounds [7] or by their spontaneous polymerization in the presence of oxygen or other oxidants, usually at alkaline pH [8]. If polymerized with other, non-aromatic precursors (proteins, peptides, amino acids, and several carbohydrates and amino sugars peptides or carbohydrates), the resulting preparations possess a resemblance to natural HS. Unlike direct labeling, these techniques, however, do not allow producing labeled HS completely identical to the native ones.

Some methods of direct labeling of HS were therefore developed, including labeling with ^{99}Tc [9] and ^{111}In [10]. The main advantage of the direct labeling of HS is an opportunity to produce a broad spectrum of isotope-labeled native humics varying significantly in both their origin and properties. Slight modification of HS functional groups was applied for introduction of halogens into HS. The method was used to radiolabel the carbon backbone of HS with ^{18}F *via* azo coupling using a precursor (4-[^{18}F]fluorobenzenediazonium ion) [11] and ^{125}I using a precursor 3-[^{125}I]iodobenzenediazonium ion [12]. The radiohalogen labeled group can react with the polyphenolic groups in the HS. The increased effort required to make the precursor is compensated for by the production of a more selective and specific label.

The state of art in ^{14}C-labeling of HS was described in Ref. [13]. Natural and synthetic humic substances were radiolabeled by azo coupling [U-^{14}C]phenyldiazonium ions onto the aromatic fragments of their macromolecules under mild reaction conditions. The chemical yields were in the range of 23 to 95%, and the specific radioactivity varied between 68 and 206 MBq/g of the humic substance, depending on the origin of the humic substance and the purification method.

Several authors suggested the use of tritium as labeling agent for humic materials [14, 15]. In Ref. [16] the method for labeling natural organic matter (NOM) with tritium (^{3}H) using fulvic acid (FA) as the target NOM fraction is described. During labeling, FA ketone groups are chemically

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*Author for correspondence (E-mail: badunga@yandex.ru).

reduced with tritiated sodium borohydride ($NaBH_4$), while the chemical functionality of the carboxyl and phenol groups is preserved. This method allowed to produce FA of high specific activity (70 GBq/g).

In this work, we report the results of radiolabeling of HS with tritium by the thermal activation method. This method is based on treatment of solid target (77 K) by hot tritium atoms, which are formed on tungsten filament (1500–2000 K) by dissociation of tritium molecules:

Introduction of tritium by the thermal activation method was demonstrated not to change structure or functional activity of labeled biomolecules [17–19] so it can be applied to different classes of compounds. Among the methods of tritium introduction into organic molecules, tritium thermal activation method is the only that allows introduction of ^3H-label in any structural fragment of macromolecule, regardless of its nearest surrounding [20]. Previously it was shown that in all organic components of HS tritium could be introduced by tritium thermal activation method. This technique was successfully applied to tritium labeling of proteins [21], polysaccharides [22] and synthetic block copolymers [23, 24].

2. Experimental

2.1 Isolation of humic substances

Humic materials were isolated from soil, peat and brown coal and included samples of humic acids (HA), fulvic acids (FA) and hymatomelanic acids (HMA). Coal humic acid (CHA-Pow) was a commercially available preparation Powhumus (Humintech GmbH, Germany) desalted using dialysis before the experiments. Coal hymatomelanic acids CHM-Pow and CHM-GL02 were obtained from the samples of CHA-Pow and HA of brown coal of Gusinoe Lake Deposit (Aginsky Buryatsky Autonomy, Russia) respectively, by ethanol extraction from freshly precipitated HA.

Peat humic materials were isolated from the samples of highland peat (Tver region, Russia) and lowland peat of Sakhtysh Lake deposit (Ivanovo region, Russia). Isolation procedure used was that described elsewhere [25] and included preliminary treatment of a peat sample with an ethanol-benzene mixture (1 : 1, v/v) followed by an alkaline extraction with 0.1 M NaOH, and acidification of the extract to pH 1–2 with 0.1 M HCl. To isolate FA, after precipitation of HA the supernatant was passed through the Amberlite XAD-2 resin as described elsewhere for isolation of the aquatic HS [26].

A sample of soil humic acids (SHA-Ctl00) was isolated from typical chernozem (related to the Mollisols) sampled nearby Lipetsk, Russia. The HA extraction was performed as described in Ref. [27]. That included preliminary treatment of soil with 0.1 M H_2SO_4 and alkaline extraction with 0.1 M NaOH, followed by acidification of the extract to pH 1–2. The precipitated HA were desalted by dialysis.

Soil fulvic acids were extracted from sod-podzolic soil (related to Spodosol) sampled near Moscow, Russia (SFA-Pg96) and typical chernozem (related to the Mollisols) sampled near Lipetsk, Russia (SFA-Ctl00). Samples of soil fulvic acids were extracted from the supernatant analogously to the samples of soil FA.

IHSS standard of fulvic acids used in the study was assigned as SRFA.

2.2 Structural characterization of HS

Elemental analyses (C, H, and N) were conducted on a Carlo Erba Strumentazione analyzer (Carlo Erba, Italy). Ash content was determined manually. Oxygen contents were calculated as a difference. The contents of all the elements were calculated on ash-free basis.

Size-exclusion chromatography (SEC) analysis was performed according to the procedure described in Ref. [28]. SEC system Abimed (Gilson, France) included HPLC pump, auto sampler, and glass column and was equipped with UV detector. The column 25 mm × 20 cm packed with Toyopearl TSK HW-55S gel (Toso Haas, Japan) was used for separation. The 0.028 M phosphate buffer (pH 6.8) was used as a mobile phase. The UV-absorbance of eluate was detected at 254 nm. Sodium salts of polystyrenesulfonic of molecular weights of 2.29, 4.48, 14.0, 20.7, 45.1, and 80.8 kDa (Polymer Standard Service, Germany) were used as markers for molecular weight calculations. HS solutions were set at a concentration of 40 mg/L by equilibrating with the mobile phase prior to the analysis. The chromatograms were analyzed as discussed in [28] to calculate the weight-average molecular weight (M_W).

Quantitative ^{13}C solution-state NMR spectra were recorded on a Varian VXR-400 spectrometer operating at 100 MHz. A weight of HS sample of 100 mg was dissolved in 3 mL of 0.1 M NaOD and transferred into a 10-mm NMR tube. All the spectra were recorded at 4-s delay time using inverse gate decoupling. These conditions were shown to provide quantitative determination of carbon distribution among the main structural fragments of HS [29]. The assignments were as follows (in ppm): 5–108 – aliphatic non-substituted and O-substituted C atoms (ΣC_{Alk}), 108–165 – aromatic non substituted and O-substituted C-atoms (ΣC_{Ar}), 165–187 – C atoms of carboxylic and esteric groups (ΣC_{COO}), 187–220 – C atoms of quinonic and ketonic groups ($\Sigma C_{C=O}$).

The obtained properties of the HS used are presented in Table 1.

2.3 Radiolabeling of HS with tritium

HS were labeled with tritium by the thermal activation method according to the procedure described in Ref. [30]. Briefly, 1 mL of HS solution (0.3 mg/mL) in 0.005 M NaOH was uniformly distributed on the wall of the cylindrical reaction vessel and lyophilized. The diameter of the vessel was 6 cm and the surface of the walls that was covered with the target was 150 cm^2. The reaction vessel with HS target was vacuumed and then filled with tritium gas to the pressure of 0.5 Pa. Tungsten filament disposed in the central part the of reactor vessel was heated by electric current to 1950 K. HS were treated by tritium atoms for 10 s, then the residual gas was pumped out. HS target was diluted in 1 mL of 0.5% NaOH. The radioactivity of solutions of labeled substances was measured by the liquid scintillation spectrometer RackBeta 1215 (Finland).

Table 1. Properties of HS used in the study.

HS index	Elemental composition			Ash, %	M_W, kD	Content of carbon in the structural fragments, %			
	H/C	O/C	C/N			$\Sigma C_{C=O}$	ΣC_{COO}	ΣC_{Ar}	ΣC_{Alk}
CHA-Pow	0.87	0.50	53	7.8	9.4	5.7	19.0	62.7	12.5
CHM-GL02	0.82	0.50	15	8.9	6.7	4.4	17.1	51.5	24.4
CHM-Pow	0.95	0.65	57	2.7	5.7	12.5	16.5	43.5	27.5
PFA-Sk3-00	1.18	0.89	27	11.2	9.2	3.2	16.9	30.0	50.0
PFA-T598	1.02	0.67	28	0.8	7.1	3.0	15.2	32.4	49.4
PHA-Sk3-00	1.15	0.66	17	3.3	18.6	1.9	16.5	34.3	47.3
PHA-T598	0.93	0.50	57	4.4	12.8	2.3	12.8	38.9	46.0
SFA-Ctl00	0.81	0.52	16	8.9	11.8	2.6	21.4	23.4	52.6
SFA-Pg96	0.88	0.61	19	5.7	11.3	3.1	18.0	41.3	37.2
SHA-Ctl00	0.79	0.35	14	6.0	15.9	3.2	15.6	52.2	27.0
SRFA	1.38	0.33	50	0.6	7.6	5.0	17.0	57.0	22.0

2.4 Purification of labeled HS

Dialysis against 0.028 M phosphate buffer (pH 6.8) with membrane of 2000 or 12 000 MWCO was used to remove tritium from labile positions (OH-, COOH- and NH_n-groups) of HS. The dialysis was carried out at 4 °C. Outer solution was periodically replaced with fresh buffer and its radioactivity was measured by liquid scintillation method.

The amount of tritium in the form of H^3HO in the external buffer was estimated. For this purpose an aliquot of external buffer was dried off and then the residue was diluted in distilled water followed by measurement of radioactivity. The radioactivity was assigned to low molecular weight fractions of HS, and the difference between the total radioactivity of the external buffer and the one related to low molecular fractions of HS corresponded to H^3HO.

2.5 Analysis of the labeled HS

^{3}H-HS samples were subjected to SEC analysis according to the procedure described above. Both UV- and radioactivity detection were applied. To register radioactivity profiles of ^{3}H-HS, 2 mL fractions were collected during the SEC experiment and analyzed for radioactivity. The yield of ^{3}H-HS was calculated as a ratio of the mass of sample in analysis determined from the UV-profile to the target mass.

3. Results and discussion

When tritium is introduced into HS it is necessary to prove that the labeled product is identical to the original HS. It is also important to determine the extent of selectivity of labeling. Thus first we will focus on the characteristics of labeled HS. Then the efficiency of HS labeling will be discussed.

3.1 Characterization of ^{3}H-HS

Interaction of atomic tritium with organic molecules leads to nonselective substitution of ^{1}H to ^{3}H in any position. Therefore it is necessary to purify the labeled compound from tritium in labile positions. Such purification is possible by multi-repeated procedure solution-lyophilization of samples. But this technique was unacceptable for HS because of possibility of sample degradation.

In this work the dialysis against 0.028 M phosphate buffer (pH 6.8) with membranes of MWCO 2000 and 12 000 were used for purification of ^{3}H-HS. Fig. 1 shows the kinetics of tritium removal from ^{3}H-HS during dialysis. The results obtained show that radioactivity of ^{3}H-HS solution noticeably decreased during fist 5 d and then the rate of tritium loss became significantly slower. Radioactivity of ^{3}H-HS was reduced to 20%–30% of initial value after 30 d of purification and it did not vary considerably in period of 30–40 d of dialysis. Thus, 30 d of dialysis purification leads to entire removal of tritium from the labile positions of labeled molecules. Such long-run dialysis of ^{3}H-HS is explained by the structural peculiarities of humic macromolecules and restricted accessibility to hydrogen exchange with water of some functional groups.

Long-run dialysis also allowed purification of tritium labeled samples from radioactive low molecular weight byproducts. Thus, the possibility of depletion of labeled samples by low-molecular fractions might occur. The ratio be-

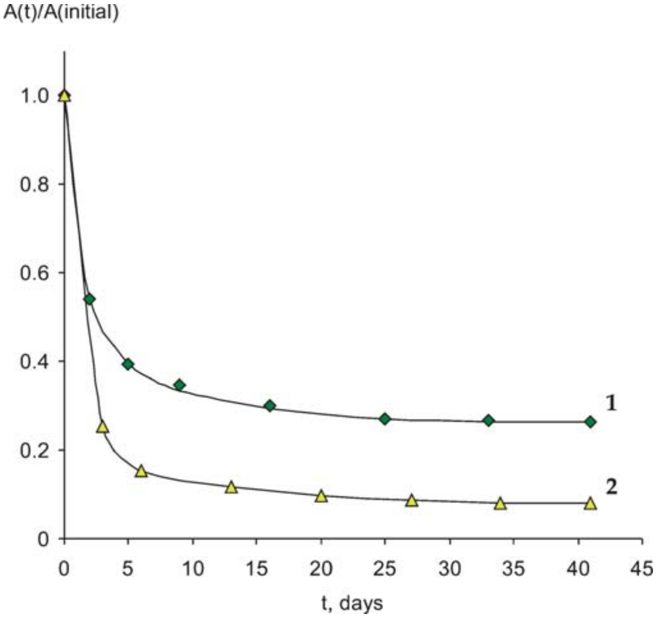

Fig. 1. The kinetics of tritium removal from ^{3}H-HS during dialysis of purified ^{3}H-HS using membrane of MWCO 2000 Da (1) or 12 000 Da (2). $A(t)$ is radioactivity of HS at time t, A(initial) is radioactivity of HS after tritium labeling.

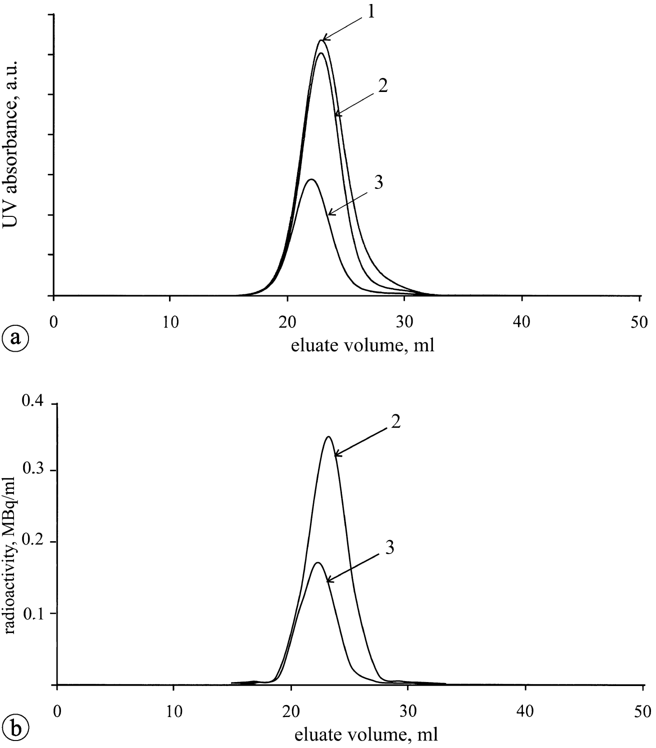

Fig. 2. SEC-profiles of initial and tritium labeled coal HA CHA-Pow with UV (**a**) and radioactivity (**b**) detection. Parent HS (1), ^{3}H-HS purified using membrane of MWCO 2000 (2) or 12 000 Da (3).

tween H^3HO (labile tritium) and low molecular weight radioactive fractions was calculated from changes of radioactivity of external dialysis solutions after its evaporation and following dilution of residue in water. This ratio changed from 10 : 1 (first day) to 1 : 10 (after 25 d). The latter finding might indicate opportunity of changes of molecular weight distribution of HS due to loss of low molecular weight fractions.

Therefore comparative analysis of parent HS and ^{3}H-HS was performed using SEC. The latter allowed monitoring both molecular weight changes in ^{3}H-HS compared with the parent humics and the distribution of tritium among the HS fractions of different molecular sizes. Typical SEC-profiles of the parent and ^{3}H-HS on the example of CHA-Pow are presented in Fig. 2a. Chromatograms of both parent HS and ^{3}H-HS exhibited single coincident peaks. Thus, one can conclude that no significant changes in HS molecules occurred during the reaction with atomic tritium.

However, when membrane of MWCO 12 000 was used for dialysis, the peak of ^{3}H-HS samples was shifted to the lower values compared to parent humics (Fig. 2a, line 3). The reason of the observed phenomenon seemed to be a depletion of the labeled samples with low molecular weight fractions. The membrane of MWCO 12 000 could therefore not be recommended for HS purification if it is needed to keep the molecular weight distribution of ^{3}H-HS. On the other hand, there was no significant change in ^{3}H-HS UV-profile compared to the parent HS when the membrane of MWCO 2000 was used (Fig. 2a, line 2).

Typical SEC profile of radioactivity of eluate is presented in Fig. 2b. SEC profiles showed that the entire eluted radioactivity was associated only with HS. UV- and radioactivity SEC profiles of ^{3}H-HS samples were the same. It has to be noted that there was shift in radioactivity-profile as well as UV-profile when membrane of MWCO 12 000 was used. Specific radioactivity of ^{3}H-HS purified by different dialysis membranes was the same (see Table 2, the data for PFA-T5H-98 and PHA-T5H-98). So we can conclude that tritium is uniformly distributed among HS fraction of different molecular sizes. Thus the developed method of tritium labeling of HS allows to obtain ^{3}H-HS with uniformly distributed tritium and identical properties to the parent HS if membrane of MWCO 2000 are applied for purification.

Table 2. Tritium labeling efficiency of humic substances.

HS	MWCO membranes	Specific activity, TBq/g	Yield, %
CHA-Pow	2000	0.33 ± 0.12[a]	84 ± 7[a]
CHM-GL02	2000	0.29	23
CHM-Pow	2000	0.28	24
PFA-Sk3-00	2000	0.17	82
PFA-T5H-98	2000	0.18	61
PFA-T5H-98	12 000	0.18	24
PHA-Sk3-00	2000	0.63	85
PHA-T5H-98	2000	0.28	94
PFA-T5H-98	12 000	0.26	61
SFA-Ctl-00	2000	0.14	85
SFA-Pg-96	2000	0.35	75
SRFA	2000	0.22	51

a: Average value from 5 experiments.

3.2 Efficiency of labeling ^{3}H-HS

There are several conditions that influence the specific activity of ^{3}H-HS: tritium gas pressure, mass of the target, temperature of the tungsten filament, the reaction time [31–33]. In this study, some of the above conditions for HS labeling were chosen based on the main appropriateness of tritium thermal activation method. In particular, applied gas pressure was *ca.* 0.5 Pa as the best labeling results were obtained for the other organic molecules under such pressure. The latter resulted from direct path of atoms from the wire to the target [34].

Another important parameter is the target mass, which was equal to 0.3 mg for HS labeling. In primary experiments it was shown that such a mass provides reproducible results. An increase in the mass of the target does not lead to a substantial increase in initial radioactivity of HS, while specific radioactivity after purification (*i.e.* final) of ^{3}H-HS was decreased [35]. The reaction time was established as 10 s as minimum by-products and high radiochemical yield were achieved [36]. The influence of wire temperature was examined for free amino acids [37]. Very high specific radioactivity and yield of the labeled product were obtained at the interval 1800–1900 K. According to primary results HS reveal approximately the same tendency [36]. The possibility of by-products formation increases when temperature is about 2000 K. Thus we chose 1950 K as an optimum wire temperature for tritium labeling of HS.

The reliability of labeling under the conditions described above was first examined on CHA-Pow and then applied to the other humics. The characteristics of tritium labeled HS are summarized in Table 2.

The obtained values of specific radioactivity of the ^{3}H-HS varied in the range 140–630 GBq/g. It has to be emphasized that such specific activities have never been achieved by other methods of tritium labeling of HS. For example, when tritium was introduced into peat HA by –CHO-groups reduction with KB^{3}H$_4$, the specific radioactivity was around 4 kBq/g [15]. The values of specific radioactivity presented in Ref. [16] seem to be the most close to our results. In the case of a reaction with tritiated NaBH$_4$, the specific activity of fulvic acid was 70 GBq/g. This procedure, however, is not applicable for humic acids. Furthermore, some modification (reduction of ketone groups of HS) occurred that cause changes in HS properties. So, labeling described above depends on an effective reactive group concentration and is a highly specific reaction [38]. In contrast to reduction with KB^{3}H$_4$, tritium thermal activation technique leads to non specific interaction of tritium with HS. It allows to introduce label in both humic and fulvic acids.

For compiling a set of structural data to be used for the correlation analysis, the approach was undertaken as described in detail in our previous publication [39]. It implies a numerical description of the structure of HS in terms of composition using a combination of the molecular descriptors of elemental, fragmental and molecular weight composition. The atomic ratios (H/C, O/C, C/N) were used as the descriptors of elemental composition, percentages of carbon in the main structural groups ($\Sigma C_{C=O}$, ΣC_{COO}, ΣC_{Ar} and ΣC_{Alk}) were used as the descriptors of fragmental composition, and the weight-averaged molecular weight (M_W) was used as a descriptor of the molecular weight composition. The corresponding data are summarized in Table 1 of experimental section.

The performed analysis revealed that no significant correlation between specific radioactivity of ^{3}H-HS and their chemical-physical properties was observed. This observation appeared a good additional argument of non-specific ^{3}H-HS distribution among initial substance.

4. Conclusions

In the present study, a new technique for radioactive labeling of HS using thermal activation method has been developed. The method implied bombardment of the target HS with atomized tritium followed by equilibrium dialysis to purify the labeled product from labile tritium and low molecular weight fractions.

To prove this technique, nine samples of different origin and fraction composition were labeled with tritium. Target HS included coal, peat and soil fulvic, humic, and hymatomelanic acids. Parent HS and obtained samples of ^{3}H-HS was analyzed by SEC with radioactivity and UV detection. The most appropriate conditions of tritium introduction into HS and purification of ^{3}H-HS were chosen based on the data obtained and were as follows: target mass 0.3 mg, treatment time 10 s, tritium gas pressure 0.5 Pa, and temperature of tungsten filament 1950 K. Purification of ^{3}H-HS should be performed using membrane with MWCO 2000. Parent and labeled HS was demonstrated to be characterized with coincident UV SEC profiles what was evident for the absence of significant alteration of HS structure due to either tritium introduction or dialysis. On the other hand, similarity of UV and radioactivity SEC profiles of ^{3}H-HS confirmed that introduced tritium was uniformly distributed among HS fractions of different molecular sizes.

In contrast to other methods of tritium introduction into organic molecules, this technique is applicable for direct labeling of different types of humic materials: humic acids, fulvic acids and hymatomelanic acids. The obtained labeled HS may be useful for the study of HS behavior in the environment and biological systems [40, 41].

Acknowledgment. We gratefully acknowledge V. Yu. Pozdnyakova and E. Yu. Belyaeva (Moscow State University) for providing help in the experiments.

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