Proc. Estonian Acad. Sci. Chem., 1994, 43, 3, 110—117
https://doi.org/10.3176/chem.1994.3.07

ELECTRODIALYSIS OF HUMIC ACIDS ISOLATED FROM THE CURATIVE MUDS OF HAAPSALU BAY AND DETERMINATION OF BUFFERING GROUPS

Sulev PIHLAK [a], Jaak ARRO [b], and Arkadi EBBER [c]

[a] Firma «Vagos» (Vagos), Sütiste tee 17, EE-0034 Tallinn, Eesti (Estonia)
[b] Eesti Veeteede Amet (Estonian National Maritime Board), Tartu mnt. 13, EE-0001 Tallinn, Eesti (Estonia)
[c] Eesti Teaduste Akadeemia Keemia Instituut (Institute of Chemistry, Estonian Academy of Sciences), Akadeemia tee 15, EE-0026 Tallinn, Eesti (Estonia)

Presented by Ü. Lille

Received March 1, 1994; accepted April 21, 1994

Abstract. Humic acids (HA), isolated from the curative muds of Haapsalu Bay, possess certain anti-inflammatory properties. The purification of these HA by using electrodialysis is described. The contents of acidic and basic buffering groups are estimated by interpreting the titration curves of the HA. The p*K*s of the buffering groups are estimated and the implications of the acquired results for the structure of the HA are discussed.

Key words: curative mud, humic acids, electrodialysis, isoelectric focusing.

INTRODUCTION

It is well known that humic acids (HA) do not exist in pure form in nature, but always contain other compounds. Inorganic salts are bound to the HA by ionic bonds and organic molecules are linked to them either by ionic bonds or by the hydrophobic interaction. Therefore, the HA must be thoroughly purified prior to analysis. Although the attempts to free the HA from inorganic salts and from other low-molecular-weight compounds by using ion-exchange resins [1], dialysis [2–5], microfiltration [6], sequential washing with strong acids and deionized water [7], and gel filtration [8] have been quite successful, some problems still remain. Sample losses cannot usually be avoided and they depend on the purification method (see ref. [9]), and often the removal of organic anions bound to HA by hydrophobic interaction, may not be complete.

The purification of very large HA molecules (molecular mass reaching up to millions of daltons) with conventional methods appears to be especially troublesome. We have reason to believe that electrodialytic methods would allow considerably to improve the purification quality of the HA. Due to the applied electric field, there should be no traces of charged particles contaminating the HA matrix after the run. In addition, the electrodialytical purification methods would also allow to obtain new information about the isoelectric properties of the HA and of their structure.

The HA subjected to electrodialysis were isolated from the curative muds of Haapsalu Bay, which have long been used in the treatment of rheumatism and other chronic inflammatory diseases [10]. 0.01% solutions of these HA in isotonic solution are distributed under the trade mark Humisol, which is an effective injection drug against chronic inflammatory diseases [11, 12]. Experimental data indicate that HA are the main constituent of Humisol and the molecular mass of these HA is reaching up to millions of daltons [6, 13].

EXPERIMENTAL

Humic acids. 0.5% stock solution of HA was obtained from the Tallinn Pharmaceutical Plant (Tallinna Farmaatsiatehas). The preparation of this solution includes the following steps:
— alkaline extraction
— separation from insoluble material by centrifugation (1800 g during 30 min)
— neutralization
— precipitation of HA in 50% ethanol solution
— separation of the precipitate by centrifugation
— dissolution of the precipitate in water
— sterilization at 100 °C

Sodium hydroxide. Sodium hydroxide was purified from contaminating anions by electrodialysis, using the same equipment that was used in the electrodialysis of HA.

Electrodialytic equipment. Electrodialysis was performed in an U-tube. The electrodialysis unit was assembled in the following order:
— 0.5% stock solution of the HA was transferred into the U-tube (length — 35 cm; internal diameter — 15 mm)
— glass tubes were fitted into the ends of the U-tube using rubber gaskets until they reached the surface of the HA solution; both glass tubes had a fritted disc inside that divided the tube into two compartments
— the upper compartments of the glass tubes were filled with distilled water
— the air that was trapped below the fritted discs was sucked out using a syringe
— electrodes were dipped into the glass tubes (anode — platinum wire; cathode — stainless steel wire)
The electrodes were connected to LKB 2103 power supply.

Electrodialysis of humic acids. Moderate voltages were used in the electrodialysis of HA in order to avoid excess heating of the sample and to reduce the electroendosmotic flow. The electric current was adjusted to 5 mA while the voltages remained between 0.1—0.2 kV. After about an hour the HA began to concentrate at the anodic side of the U-tube, but they did not migrate through the fritted discs into the anodic compartment. A transparent zone started to emerge below the fritted disc of the cathodic compartment. Excess liquid was removed occasionally from the cathodic chamber and distilled water was added into the anodic chamber.

The electrodialysis was continued for two days (interrupted at night) until a sharp boundary emerged between the dark HA zone and the transparent zone of alkaline water solution, which both occupied about 50% of the volume of the U-tube. Then, the electrodialysis was discon-

tinued and the unit was carefully disassembled in order to avoid mixing, and the alkaline water solution (pH 9.3) as well as the anolyte (pH 1.6) and catholyte (pH 12.4), were removed. pH of the HA fraction that remained in the U-tube was 2.8.

The liquid level in the U-tube was readjusted with distilled water, the glass tubes were again fitted into the U-tube and the run was continued in order to remove the possible traces of contaminants from the HA solution. Due to the reduced conductivity, the voltage between the electrodes increased sharply and strong electroendosmotic flow towards the cathode occurred. The voltage limit was set to 1 kV (the current was below 1 mA) and the run was continued for two more hours, constantly removing excess liquid from the cathode and pipetting distilled water into the anodic chamber. The HA did not concentrate at the anodic side of the U-tube as they did before the removal of the alkaline water solution, but occupied the whole volume of the U-tube, although the slurry was much more dense in the bottom of the U-tube.

After the run, the HA slurry was transferred into a beaker and the remaining transparent liquid was removed. The slurry was then homogenized with a microstirrer and a sample of it was quickly weighed into a beaker and dried to constant weight at 105 °C air thermostat in order to estimate the dry matter content. Another sample from the homogenized slurry was weighed into the titration beaker.

Elemental contents of the three different batches of HA subjected to microfiltration and electrodialysis are presented in Table.

Elemental composition of humic acids after the application of different purification methods

Batch No and purification method	Elemental composition		
	C (std)	H (std)	N (std)
Batch No 1, Oct. 1990			
Without purification	35.3 (0.4)	4.85 (0.12)	2.92 (0.02)
Microfiltration	39.5 (0.3)	5.13 (0.15)	3.43 (0.05)
Electrodialysis	43.2 (0.2)	5.49 (0.01)	3.74 (0.02)
Batch No 2, Sept. 1991			
Without purification	32.6 (0.4)	4.44 (0.13)	2.66 (0.00)
Microfiltration	39.2 (0.2)	5.31 (0.14)	3.25 (0.04)
Electrodialysis	44.0 (0.5)	5.90 (0.27)	3.69 (0.09)
Batch No 3, Oct. 1992			
Without purification	31.4 (0.2)	4.20 (0.22)	2.58 (0.02)
Microfiltration	39.4 (0.1)	5.08 (0.06)	3.28 (0.04)
Electrodialysis	44.4 (0.2)	6.02 (0.18)	3.80 (0.00)

Microfiltration. 200 nm Mifil membranes were from Himfil, Estonia. Microfiltration of 10 ml 0.5% stock solution of HA was performed in a filtration cell equipped with magnetic stirrer under 2 Bar argon pressure. After filtering about 90% of the solution, distilled water was added on the filter cake up to the initial level and the filtration was repeated in order to remove dialyzable contaminants from the HA. This procedure took about 45 min and it was repeated four times. The contents of the

dry matter in the final permeate was about 0.1 mg. Over 30% of the dry matter content from the HA stock solution permeated the membrane during the filtration and washing steps (see also ref. [6]).

Elemental analysis. Carbon, hydrogen and nitrogen contents were analysed using the Hewlett-Packard 186 CHN analyser.

pH measurements and titration of humic acids. Digital burette KB1 (VEB MLW, GDR) was equipped with polyethylene capillary tube that was dipped directly into the titrated solution in order to improve the titration accuracy. pH was measured with digital pH-meter OP-11/1 (Radelkis, Hungary), equipped with combined electrode OP-0808P (Radelkis).

The HA solution (0.141 g of dry matter in approx. 40 ml of solution) was titrated with 442 mM NaOH in a beaker equipped with magnetic stirrer and with an argon inflow. Before the titration, 0.234 g NaCl was transferred into the HA solution (0.234 g NaCl in 40 ml corresponds to 0.1 M NaCl). In blank titration all conditions were the same, except that the HA were absent and the pH of the solution was adjusted to the value of 2.73 with 3 M HCl. The results of the titrations are presented in Fig. 1.

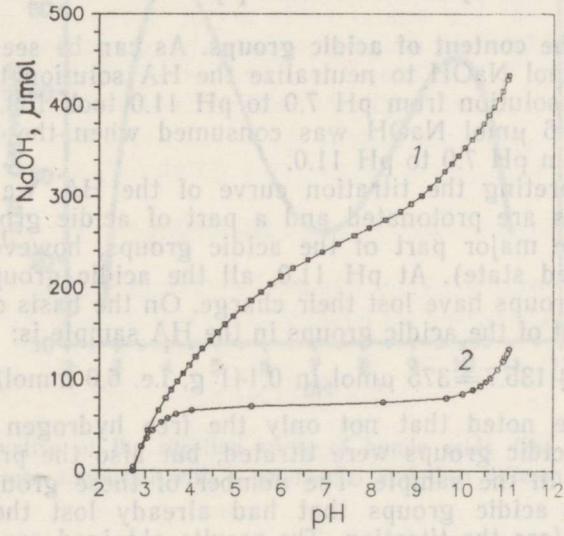

Fig. 1. Titration of humic acids with 442 mM NaOH. *1* — 0.141 g humic acids in 40 ml 0.1 M NaCl; *2* — blank titration.

DISCUSSION

Similarity to isoelectric focusing. Although the electrodialysis was used with the objective of obtaining pure HA for titration, additional conclusions can be drawn from the experimental data. In fact, the mechanism of the electrodialysis of HA is rather similar to that of the isoelectric focusing. HA that are charged in the beginning of the run migrate in the electric field until they lose their charge. pH gradient, however, is not generated by synthetic ampholytes, but by the HA

molecules themselves as they contain both acidic and basic buffering groups. pH of the electrodialytically purified HA sample should, therefore, indicate the mean value of the isoelectric points of the sample. It is accepted that the sedimentary HA are complex mixtures of large molecules [14, 15], and there must also be some variances in the p*I*s of the individual molecules, but the mean value of the isoelectric points seems to be well below the pH 2.73. This pH was generated by a dilute solution of HA (0.141 g in 40 ml, i.e. 0.35%). Another characteristic that makes it difficult to measure the p*I* of the HA is their very weak buffering power at this pH region.

Earlier, an attempt was made to analyse the HA by applying the techniques of conventional isoelectric focusing (HA samples were run in polyacrylamide and agarose gels, using synthetic ampholytes). These methods, however, were not very accurate as the HA molecules were too large for the gel electrophoresis [6].

Application of different purification techniques. In comparison with microfiltration, purification by electrodialysis seems to be more effective when contaminating cations and anions are to be removed from the HA solution since the contents of carbon, hydrogen and nitrogen were significantly higher after the application of electrodialysis (Table). Purification of HA by the methods of conventional dialysis has been about as effective as by microfiltration [4].

Estimation of the content of acidic groups. As can be seen from Fig. 1, it took 239.3 μmol NaOH to neutralize the HA solution to pH 7.0. The titration of the solution from pH 7.0 to pH 11.0 took 189.3 μmol NaOH respectively. 53.6 μmol NaOH was consumed when the blank solution was titrated from pH 7.0 to pH 11.0.

While interpreting the titration curve of the HA, we presume that all basic groups are protonated and a part of acidic groups is ionized at pH 2.73 (the major part of the acidic groups, however, remains in the undissociated state). At pH 11.0, all the acidic groups are ionized and the basic groups have lost their charge. On the basis of the acquired data, the content of the acidic groups in the HA sample is:

$$239.3+135.7=375 \text{ μmol in } 0.141 \text{ g, i.e. } 6.0 \text{ mmol/g C.}$$

It should be noted that not only the free hydrogen ions and the undissociated acidic groups were titrated, but also the protonated basic groups present in the sample. The number of these groups is equal to the number of acidic groups that had already lost their protons to basic groups before the titration. The results obtained are in accordance with the available data about the bottom sediments of the Baltic Sea where the average contents of carboxylic groups in HA are ranging from 2.0 to 7.2 mmol/g C [16].

Estimation of p*K* values. Fig. 1 shows that 298 μmol NaOH was consumed for the deprotonization of acidic and basic groups from pH 2.73 to pH 11.0. In order to determine the mean p*K* values of the acidic and basic groups present in the HA, blank titration was eliminated from the HA titration curve (Fig. 2). Experimental data were interpolated by cubic spline and then subtracted. The graph in Fig. 2 was simulated by a polynomial of the 7th power and the first derivative of that polynomial is presented in Fig. 3. It can be seen that there are two maxima in the derivative graph. Since the shape of the peaks indicates that the distribution of p*K*s is close to the normal distribution, the maxima of the peaks may be interpreted as the mean p*K* values [17].

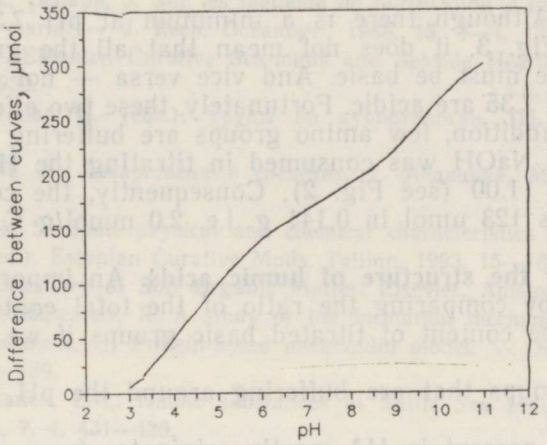

Fig. 2. Titration curve of the humic acids after the elimination of blank titration.

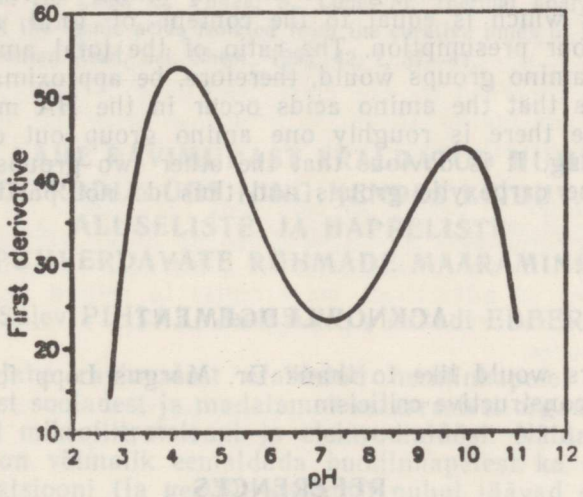

Fig. 3. First derivative of the titration curve of humic acids (the titration curve in Fig. 2 was simulated with a polynomial and then differentiated).

One of the maxima is at pH 4.20, which is the characteristic pK of carboxylic groups. For soil HA, for example, the mean pK values of the carboxylic groups have been estimated to range between 3.7 and 4.7 [17]. The smaller maximum at pH 9.85 is in very good agreement with the characteristic pK of amino groups. Although it is known that phenolic hydroxyl groups are also buffering in that region, our previous results indicate that the presence of aromatic structures in the HA of Humisol is negligible [18].

The calculations were performed using the software package 386-MATLAB (The MathWorks, Inc.).

Estimation of the content of basic groups. On the basis of our data, it is also possible to estimate the content of basic groups present in the HA. The result, however, would not be as accurate as in the case of

acidic groups. Although there is a minimum at pH 7.35 between the two peaks in Fig. 3, it does not mean that all the groups buffering above that value must be basic. And vice versa — not all groups buffering below pH 7.35 are acidic. Fortunately, these two effects compensate each other. In addition, few amino groups are buffering beyond the pH 11.00. 123 μmol NaOH was consumed in titrating the HA sample from pH 7.35 to pH 11.00 (see Fig. 2). Consequently, the content of basic groups in HA is 123 μmol in 0.141 g, i.e. 2.0 mmol/g C.

Implications for the structure of humic acids. An important conclusion can be drawn by comparing the ratio of the total content of nitrogen in the HA to the content of titrated basic groups if we make two presumptions:

— the basic groups that are buffering around the pH 9.85 are amino groups

— the nitrogen present in HA mostly originates from amino groups.

These presumptions are in accordance with the data about the composition of HA isolated from the curative muds of Haapsalu Bay [4] and about the sedimentary HA in general [15].

The content of total nitrogen in the dry matter of HA is 3.8%, i.e. 6.1 mmol/g C which is equal to the content of total amino groups according to our presumption. The ratio of the total amino groups to the buffering amino groups would, therefore, be approximately 3 : 1.

This means that the amino acids occur in the HA matrix in short chains because there is roughly one amino group out of every three that is buffering. It is obvious that the other two groups form peptide bonds with the carboxylic groups and thus do not participate in buffering.

ACKNOWLEDGEMENT

The authors would like to thank Dr. Margus Lopp for fruitful discussions and constructive criticism.

REFERENCES

1. Pettersson, C., Ephrain, J., Allard, B. On the uniqueness of fulvic acids extracted from different sources. — Finnish Humus News, 1991, **3**, 3, 133—138.
2. Govi, M., Montecchio, D., Ciavatta, C. Characterization of humic and humic-like substances in organic fertilizers and amendments. — Finnish Humus News, 1991, **3**, 3, 303—308.
3. Tipping, E., Backes, C. A., Hurley, M. A. The complexation of protons, aluminium and calcium by aquatic humic substances: a model incorporating binding-site heterogenity and macroionic effects. — Water Res., 1988, **22**, 5, 597—611.
4. Ilomets, T., Pärn, A., Raidaru, G., Salm, S., Veermäe, T. Humisooli keemilisest koostisest. — Eesti rohuteadlane, 1992, **III(XVIII)**, 1, 4—10.
5. Bonn, B. A., Fish, W. Variability in the measurement of humic carboxyl content. — Environ. Sci. Technol., 1991, **25**, 2, 232—240.
6. Pihlak, S., Arro, J. Isoelectric focusing of humic acids isolated from the curative muds of Haapsalu Bay. — Proc. Estonian Acad. Sci. Chem., 1992, **41**, 1, 14—17.
7. Manunza, B., Gessa, C., Deiana, S., Rausa, R. A normal distribution model for the titration curves of humic acids. — J. Soil Science, 1992, **43**, 127—131.
8. Илометс Т., Сальм С. Исследования в области пелойдных высокомолекулярных веществ гуминокислотного характера. I. — Уч. зап. Тартуск. ун-та, 1972, **302**, 88—93.

9. Boussemart, M., Benaim, J. Sur les méthode de purification d'acides fulviques issus du milieu marin. — J. Rech. Océanogr., 1988, **13**, 3—4, 132—135.
10. Schlossman, K. Estonian Curative Sea-muds and Seaside Health Resorts. Boreans, London, 1939.
11. Суй И., Круглова Н. (eds.). Труды по курортологии, **III**. Валгус, Таллинн, 1966.
12. Машковский М. Д. Лекарственные средства, 2. Медицина, Москва, 1984, 153—154.
13. Arro, J., Pihlak, S. Some physical and chemical characteristics of Humisol. — In: Arro, J. (ed.). Estonian Curative Muds. Tallinn, 1993, 15—18.
14. Kumada, K. Chemistry of Soil Organic Matter. Elsevier, Amsterdam, 1987.
15. Yamamoto, S., Ishiwatari, R. A study of the formation mechanism of sedimentary humic substances. II. Protein-based melanoidin model. — Org. Geochem., 1989, **14**, 5, 479—489.
16. Naik, S., Poutanen, E.-L. Humic substances in Baltic Sea sediments. — Oceanol. Acta, 1984, **7**, 4, 431—439.
17. Perdue, E. M. Modeling the acid-base chemistry of organic acids in laboratory experiments and in freshwaters. — In: Perdue, E. M., Gjessing, E. T. (eds.). Organic Acids in Aquatic Ecosystems. John Wiley & Sons, S. Bernhard, 1990, 111—126.
18. Arro, J., Taal, H., Lahe, L., Pihlak, S., Lopp, M. Thermal analysis and infrared spectra of the humic acids isolated from the curative muds of Haapsalu Bay. — Proc. Estonian Acad. Sci. Chem., 1993, **42**, 1, 37—41.

HAAPSALU LAHE RAVIMUDAST ERALDATUD HUMIINHAPETE ELEKTRODIALÜÜS NING NENDES LEIDUVATE ALUSELISTE JA HAPPELISTE PUHVERDAVATE RÜHMADE MÄÄRAMINE

Sulev PIHLAK, Jaak ARRO, Arkadi EBBER

Haapsalu lahe ravimudast eraldatud humiinhapete puhastamiseks anorgaanilistest sooladest ja madalamolekulaarsetest orgaanilistest ühenditest kasutati mikrofiltratsiooni ja elektrodialüüsi. Näidati, et elektrodialüüsi abil on võimalik eemaldada humiinhapetest ka need lisandid, mis mikrofiltratsiooni (ja geelfiltratsiooni) puhul jäävad seotuks. Elektrodialüüsiga puhastatud humiinhapete tiitrimistulemuste põhjal määrati happeliste ja aluseliste puhverdavate rühmade sisaldus ja nende happelisuse konstandid ning tehti järeldusi humiinhapete struktuuri kohta.

ЭЛЕКТРОДИАЛИЗ ГУМИНОВЫХ КИСЛОТ ЛЕЧЕБНЫХ ГРЯЗЕЙ ХААПСАЛУСКОГО ЗАЛИВА И ОПРЕДЕЛЕНИЕ В НИХ БУФЕРИРУЮЩИХ КИСЛОТНЫХ И ОСНОВНЫХ ГРУПП

Сулев ПИХЛАК, Яак АРРО, Аркадий ЭББЕР

Гуминовые кислоты, выделенные из лечебных грязей Хаапсалуского залива, обладают определенными антивоспалительными свойствами. Очистка гуминовых кислот от минеральных солей и низкомолекулярных органических соединений проведена методами электродиализа и микрофильтрации. Содержание буферирующих кислотных и основных групп оценивалось по кривым титрования гуминовых кислот щелочью. Определены константы кислотности pK буферирующих групп и сделаны некоторые выводы о структуре гуминовых кислот.