

Separation and characterization of humic acids from Antarctica by capillary electrophoresis and matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Inclusion complexes of humic acids with cyclodextrins

Dagmar Gajdošová^a, Klára Novotná^a, Pavel Prošek^b, Josef Havel^{a,*}

^aDepartment of Analytical Chemistry, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic

^bDepartment of Geography, Faculty of Science, Masaryk University, Kotlářská 2, 611 37 Brno, Czech Republic

Abstract

A new capillary electrophoresis procedure based on micellar electrokinetic capillary chromatography for the separation of humic acids (HAs) isolated from Antarctica soil was developed. The HAs were separated and characterized using a background electrolyte containing 0.09 M borate+0.09 M Tris+0.001 M EDTA (BTE) of pH 8.3, modified with α -, β -, or γ -cyclodextrins (CDs) and sodium dodecyl sulfate. It was found that from alkaline solution of HAs in the presence of CDs, the HAs are not completely precipitated with a strong acid and a certain part (some fractions) remains soluble. Mass spectrometry shows that HAs contain 15–25 simple low- M_r compounds and several families of compounds with similar structure ($m/z \approx 800$ –1200). Comparison of HA analysis from Antarctica soil with those of soil HAs from the American continent show a high similarity between the samples and confirm several identical compounds and some with very similar structural units.

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1. Introduction

The term soil organic matter (SOM) represents the organic part of soil. SOM includes high-molecular-mass organic material (proteins, etc.), small molecules (amino acids, sugars, etc.) and humic substances (humic acids, fulvic acids and humin).

Humic acids (HAs), natural compounds widely distributed in nature, still of unknown structure, play an important role in the environment. HAs from soil, coal, peat, plant or water are investigated by various

techniques, like mass spectrometry (MS) [1–3], nuclear magnetic resonance (NMR) [4], capillary electrophoresis (CE) [5–9], fluorescence spectroscopy [10], etc., and in spite of much effort their structures are still not completely clear. HAs and their derivatives are used for removal of toxic metals from waste water, they are applied in the paper industry for paper coloring and removing toxic substances (e.g., lead), in agrochemistry, etc.

The formation of soil organic matter depends on climate conditions, vegetation organisms, temperature, sunlight, etc. In Antarctica, the coldest continent on Earth, there are extreme environmental conditions. The majority of the Antarctic continent is

*Corresponding author. Fax: +420-5-4121-1214.

E-mail address: havel@chemi.muni.cz (J. Havel).

still covered by ice and snow. In spite of this the Antarctic ecosystem is characterized by a large number of species of non-flowering plants (mosses, lichens, algae, fungi) and animals (whales around the continent, seals, penguins, and other sea birds). Only two species of flowering plants are known: the Antarctic hair grass (*Deschampsia antarctica*) and the Antarctic pearlwort (*Colobanthis quitensis*) and together with the mosses are the main source of organic soil [11].

There are several publications concerning investigation of soil, plant, and water in Antarctica [12–15]. Recent Antarctica soil studies confirm that there are humic acids in spite of the absence of plants containing lignin [16,17]. We have proved the formation of HAs in places like South Shetland Island, King George Island where like in the whole of Antarctica there are no trees or shrubs, and only hair grass and pearlwort plants. The details of this study can be found elsewhere [17].

The aim of the present work is to improve methods for analyzing humic acids from Antarctic soil, to continue in analysis and characterization of HAs from the other part of King George Island and to compare the results with those for standard soil humic acids from other continents. The goal is also to understand better the humification process under arctic conditions and the nature of Antarctic humic acids.

2. Experimental

2.1. Chemicals and reagents

Boric acid (analytical-reagent grade), 1,1-tris-(hydroxymethyl)aminomethane (analytical-reagent grade, (Tris), hydrofluoric acid (HF), ethylenediaminetetraacetic acid (analytical-reagent grade, EDTA), sodium dodecyl sulfate (SDS), hydrochloric acid and potassium chloride were from Lachema (Brno, Czech Republic). The α -, β - and γ -cyclodextrins (CDs) and sodium hydroxide of analytical purity were purchased from Merck (Darmstadt, Germany).

2.2. Humic acids

Humic acids were extracted from Antarctica soil

(South Shetland Island, King George Island, Machu Picchu station during Antarctica's summer, in March 2002). The samples signed as HA1-2002 represent humic acid extracted from the soil collected 10–20 m from the coast. The soil at sampling places contains evidently decomposition product of *D. antarctica*. The sample HA2-2002 humic acid was extracted from soil collected 100–200 m from the coast. The soil of this distance also contains decomposition products of *D. antarctica* and mosses, as well. Details concerning the collection of 2001 samples can be found elsewhere [17].

The International Humic Substance Society (IHSS) standard soil derived HA soil IHSS standard (1S102 H, St. Paul, MN, USA) and peat IHSS standard (1S103H) were purchased from the IHSS. Coal-derived HA Chemapex standard was a gift from Chemapex (Chomutov, Czech Republic), detailed characterization of this product can be found elsewhere [8].

2.2.1. Procedure of HA extraction

Antarctica humic acids were extracted with slightly modified procedure recommended and used by the IHSS to isolate standards of humic acid [18].

The roots and big parts were removed and the sample was air dried until constant mass. The dried soil sample was sieved to pass through a 2.0-mm sieve. The sample was equilibrated to a pH value between 1 to 2 with 1 M HCl at room temperature. The solution volume was adjusted with 0.1 M HCl to provide a final concentration (10 ml liquid/1 g of dry sample). The suspension was shaken for 1 h and the supernatant was separated from the residue by a low-speed centrifugation.

The soil residue was neutralized with 1 M NaOH to pH 7.0 and 0.1 M NaOH added to give a final extractant-to-soil ratio of 10:1. The suspension was extracted by shaking for a minimum of 4 h. The alkaline suspension was allowed to settle overnight and the supernatant collected by centrifugation. The supernatant was acidified with 6 M HCl with constant stirring to pH 1.0 and then allowed to stand for 12 to 16 h. It was then centrifuged to separate the humic acid (precipitate) fraction.

The humic acid fraction was redissolved by adding a minimum volume of 0.1 M KOH. Solid KCl was added to attain a concentration of 0.3 M $[K^+]$ and then centrifuged at high speed to remove the sus-

pended solids. The humic acid was reprecipitated by adding 6 M HCl with constant stirring to pH 1.0 and allowed to stand again for 12 to 16 h. The supernatant was centrifuged and discarded. The humic acid precipitate was suspended in a mixture 0.1 M HCl–0.3 M HF solution in a plastic container and shaken overnight at room temperature. After centrifuging, the HCl–HF treatment was repeated to obtain a sufficiently low ash content. The humic acid was washed with distilled water until the water gave a negative test for chloride with silver nitrate AgNO₃. The humic acid was then dried.

2.3. Apparatus

CE analysis was performed on an Agilent ^{3D}CE system (Agilent Technologies, USA), using an uncoated fused-silica capillary of 48.5 cm (40 cm to the detector) × 75 μm I.D. The detection was spectrophotometric.

Mass spectra were measured with an AXIMA-CFR from Shimadzu (Kratos Analytical, Manchester, UK) mass spectrometer, software version V 2.2.1. The instrument was equipped with a nitrogen laser, wavelength 337 nm.

2.4. Electrophoretic procedure

The capillary was at the beginning of daily work washed for 5 min with 1 M NaOH solution, followed by 5 min washing with tridistilled water and 5 min with buffer at 25 °C.

Various background electrolytes (BGEs) are used for the separation of humic acids. In this work a mixed buffer containing 0.09 M borate, 0.09 M Tris,

0.001 M EDTA (pH 8.3) (BTE buffer) with SDS and cyclodextrins as additives was used here.

The separation voltage +20 kV, detection at 210 nm and the temperature 25 °C was used. The samples were injected by hydrodynamic injection (20 s). The capillary was post-washed for 1 min with 1 M NaOH, 2 min with tridistilled water and for 2 min with the same BGE (0.09 M borate, 0.09 M Tris, 0.001 M EDTA, pH 8.3) but not containing SDS after each analysis. In this way the results were more reproducible.

2.5. Sample preparation for mass spectrometry analysis

All HA samples prepared as described in Section 2.2.1 were dissolved in 0.036 M NaOH solution (1 mg in 1 ml 0.036 M NaOH). In order to perform MS analysis, 1 μl of such solution was dropped to a sample plate, dried in an air stream at room temperature and inserted into the vacuum chamber of the instrument. After deep vacuum was reached ($\approx 10^{-4}$ Pa) the mass spectra were measured. Always the resulting spectra were accumulated from at least 100 shots.

3. Results and discussion

HA samples obtained according to Section 2.2.1 were analyzed by elemental analysis. The result of elemental analysis of the samples and some characteristic parameters are collected in Table 1, results of metal content analysis in Table 2. It follows from Table 1 that the carbon contents of Antarctica HAS

Table 1
Elemental analysis and characteristic parameters of HA

HA sample	C (%)	H (%)	N (%)	Ash content (%)	Carboxylic acidity (mequiv./100 g)
HA1-2002	50.73 (53.8)	4.79 (5.08)	5.95 (6.3)	5.71	142.1 (150.6)
HA2-2002	51.8 (52.88)	5.37 (5.48)	5.2 (5.31)	2.05	168.9 (172.4)
IHSS soil standard	58.13	3.68	4.14	0.88	351.8 (386.9)
IHSS peat standard	56.37	3.82	3.69	1.12	–
Chemapex coal-derived standard	50.25 (54.2)	4.9 (5.3)	1.23 (1.33)	7.3	–

Data in parentheses are calculated for a dry, ash-free basis.

Table 2
Trace elements content in humic acids (mg/g HA)

HA sample	As	Cr	Zn	P	Co	Cd	Ni	Pb	B	Si	Mn	Fe	Cu	Al
HA1-2002	<0.010	0.008	0.006	5.676	0.011	<0.002	0.005	<0.010	0.480	11.085	0.002	0.920	0.077	3.303
HA2-2002	<0.010	0.008	0.009	8.108	<0.005	<0.002	0.010	<0.010	0.472	8.336	0.002	2.675	0.066	0.897
IHSS Soil standard	<0.010	0.023	0.075	5.405	0.007	<0.002	0.015	<0.010	0.362	6.070	0.002	0.786	0.256	0.553
Chemapex coal-derived standard	<0.010	0.024	0.031	0.500	–	<0.002	0.014	<0.010	–	22.200	–	–	0.028	9.140

are slightly lower than those of both soil and peat IHSS standards and is almost the same as the coal-derived Chemapex standard. The opposite is true for hydrogen and nitrogen contents. The ash contents in Antarctica HAs are also higher, which are in agreement with much higher contents of Si, Fe and Al in comparison to soil and peat standards. Levels of trace elements (As, Cr, Zn, Co, Cd, etc.) are comparable for all samples. There is also higher average phosphorus content in comparison with other samples. Higher P and N contents can be due to rather high number of penguins living around the sampling sites.

3.1. Micellar electrokinetic capillary chromatography

In the past, various buffers were used for HA separation, mostly based on borate. Also, addition of individual cyclodextrins and their mixtures on the separation of HAs was studied [8]. As the most successful background electrolyte a combination of borate with Tris and EDTA was found [9]. EDTA is complexing traces of metal ions in HAs and thus improves the separation. However, the results often show unresolved “hump” of humic acids with some peaks but not sufficiently separated. Therefore, in this work we are further trying to modify the background electrolyte and to improve the separation.

We started with the BTE buffer (as mentioned in Section 2.4), which was further modified with a mixture of SDS or α -, β - and γ -cyclodextrins. The anionic surfactant (SDS) was used in the concentration range from 0.002 to 0.1 M. The critical micelle concentration (CMC) for this surfactant is ≈ 0.008 M at 25 °C. The results of the BTE buffer modified with SDS show that only a slight effect on

the separation of humic acids is observed. In Fig. 1 examples of electropherograms of soil IHSS standard obtained using BTE buffer with 0.03 M and 0.1 M SDS are shown. It follows from Fig. 1, that prolongation of migration time of HA peaks is observed, while simultaneously the sensitivity increases. As no substantial effect of SDS was found, we have further tried to modify this buffer using various additions of α -, β -, and γ -cyclodextrins. It was found that for the background electrolyte modified with a mixture of cyclodextrins and SDS better separation patterns were observed. Concluding, modification with 0.03 M SDS+0.01 M β -cyclodextrin mixture was found best. Better separation effect can be either due to stronger complexation of β -CD with low-molecular-mass fractions present in

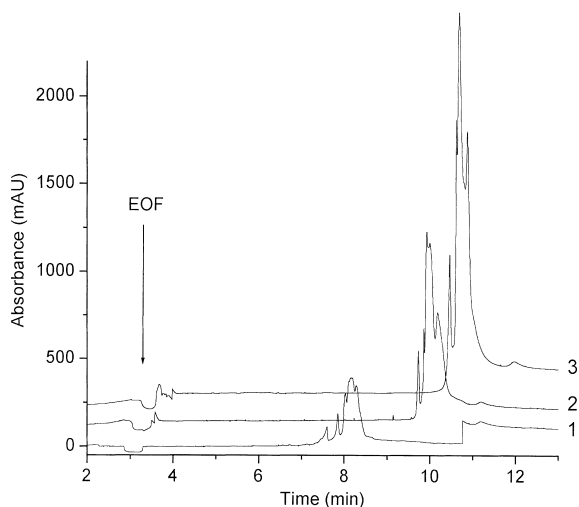


Fig. 1. Electropherograms of soil IHSS humic acid in different background electrolytes: (1) 0.09 M borate 0.09 M Tris \pm 0.01 M EDTA (BTE), (2) BTE \pm 30 mM SDS, (3) BTE \pm 100 mM SDS. Separation conditions: pH of background electrolytes 8.3, separation voltage 20 kV, hydrodynamic injection 20 s.

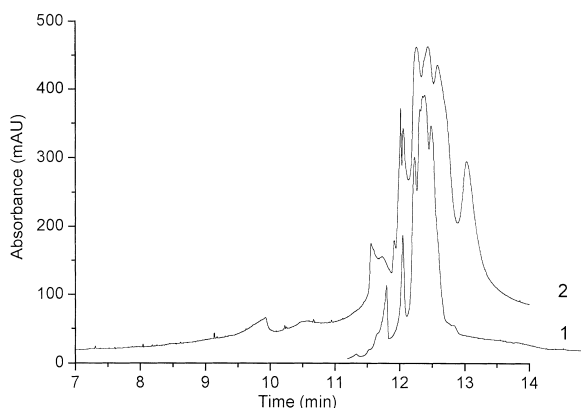


Fig. 2. Electropherograms of soil IHSS standard humic acid obtained using different background electrolytes: (1) BTE, (2) BTE+30 mM SDS+10 mM β -cyclodextrin. Separation conditions: pH of background electrolytes 8.3, separation voltage 20 kV, hydrodynamic injection 20 s.

HAs or with side chain groups of humic acids. An example of the separation pattern is given in Fig. 2. Using this buffer the samples of Antarctica HAs and soil IHSS standard were analyzed (Fig. 3). It follows from Fig. 3 that in (SDS+ β -CD) modified BTE buffer the classical “hump” of humic acids is almost separated (for HA1-2002 up to 5–7 fractions). There is an indication that also Soil IHSS standard contains

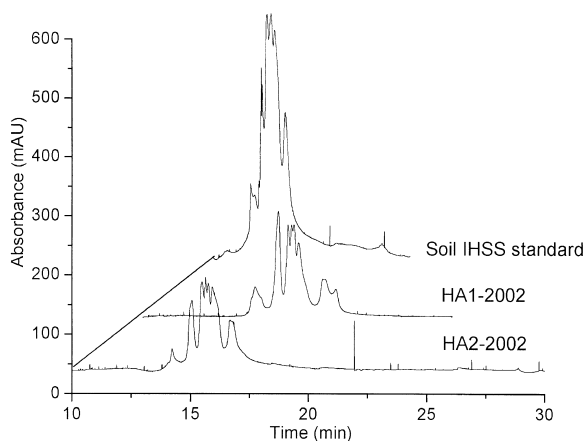


Fig. 3. Electropherograms concerning HAs extracted from Antarctica soil and soil IHSS standard analysis. Separation conditions: background electrolyte BTE+30 mM SDS+10 mM β -cyclodextrin, pH 8.3, separation voltage 20 kV, hydrodynamic injection 20 s.

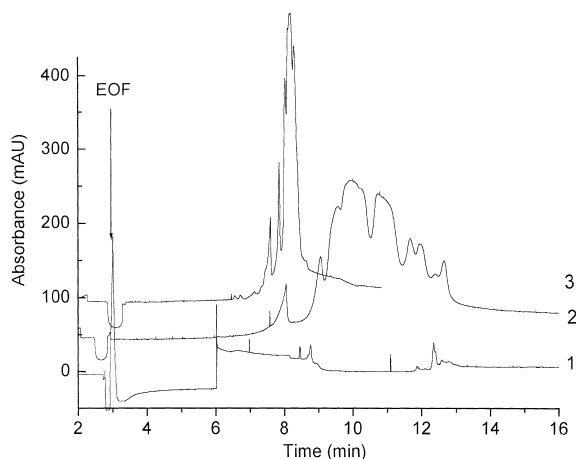


Fig. 4. Electropherograms concerning soil IHSS standard analysis: (1) fraction soluble in γ -cyclodextrin, (2) HAS “purified” with γ -cyclodextrin, (3) original soil IHSS standard. Separation conditions: background electrolyte BTE, pH 8.3, separation voltage 20 kV, hydrodynamic injection 20 s.

5–8 fractions (cf. Fig. 2, curve 2; Fig. 3, curve soil IHSS standard).

3.2. Purification of humic acids with γ -cyclodextrin

It was demonstrated above (Section 3.1) for CD-modified BGEs that the separation improved. The logical explanation is that some of HA fractions are complexed with CD, forming inclusion complexes. We have therefore tried to improve CE separation of HAs eliminating some of these compounds from HA samples. Because the solubility of β -CD is much lower than that of γ -CD, mostly γ -CD was used for the purification. In order to do it the following procedure was applied.

The solution of humic acid (1 mg/ml) to which γ -cyclodextrin was added up to 0.01 M level, was precipitated adding slowly under continuous stirring with 6 M HCl to pH \approx 1. Surprisingly, it was observed that HAs are not precipitated completely and that a certain part of HA remains soluble forming a yellow solution above the precipitate. After 10 min centrifugation the mother liquid was removed, the solid HA fraction was isolated and dissolved again in 0.036 M sodium hydroxide solution. After adding another portion of CD, the precipi-

tation procedure was repeated (six times in total). In this way obtained “purified” HA and the cyclodextrin solution containing soluble fraction were analyzed and the results compared. Fig. 4 shows electropherograms of soil IHSS standard humic acids purified with γ -cyclodextrin and the CD-soluble part and the comparison with original soil IHSS standard dissolved in 0.036 M NaOH. These samples were analyzed by CE using BTE buffer. It was found (Fig. 4, curve 1), that the HA fraction complexed with γ -cyclodextrin which is not precipitated with acid, shows several simple peaks and no typical “hump”. We can suggest that γ -CD is “extracting” some low- M_r fractions of HA. This is in agreement with MS results (cf. Section 3.3). The purification procedure was also applied to Antarctica samples. Fig. 5 shows electropherograms of purified humic acid from Antarctica HA2-2002 and comparison with humic acids extracted from Antarctica soil HA2-2002 dissolved in 0.036 M NaOH. It is evident that the CD-purified Antarctica HA sample is much better separated into 5–6 fractions (Fig. 5, curve 2). It is interesting, that for both, soil IHSS standard and Antarctica HA2-2002 sample, electropherograms of the CD-soluble part show remarkable peak quite close to the electroosmotic flow (EOF) (Fig. 4, curve 1; Fig. 5, curve 1).

Both, original and purified humic acids as well as

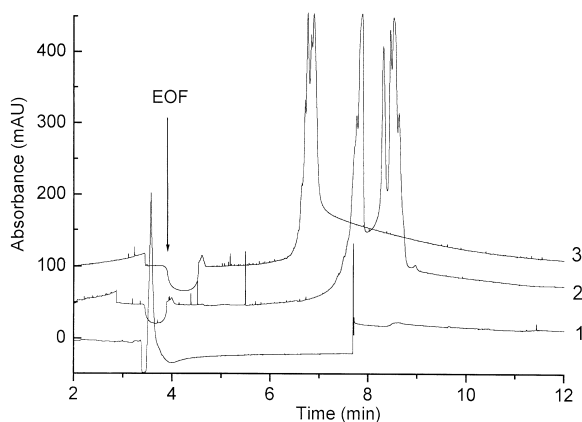


Fig. 5. Electropherograms concerning Antarctica HA2-2002 analysis: (1) fraction soluble in γ -cyclodextrin, (2) HAs “purified” with γ -cyclodextrin, (3) original HA2-2002 sample. Separation conditions: background electrolyte BTE, pH 8.3, separation voltage 20 kV, hydrodynamic injection 20 s.

the γ -CD-soluble fractions were also analyzed by MS and the results compared. Similar results were obtained for β -CD-purified humic acids.

3.3. Humic acid analysis with mass spectrometry

Mass spectra were measured on a matrix-assisted laser desorption time-of-flight (MALDI-TOF) instrument. Similarly as already described by us in several papers [8,9,17], the HA spectra were measured in the laser desorption ionization (LDI) mode without using any matrix. Using matrices (2,5-dihydroxybenzoic acid, ferrulic acid, and others) we have always observed quite complicated spectra showing that organic matrices interact with some of the HA components.

Fig. 6A shows an example of mass spectra concerning the soil IHSS standard. The part of spectra between m/z 800–950 is similar for all humic acids studied, in agreement with previous results [17]. Fig. 6B shows details of one of the peaks ($m/z \approx 878.6$). As m/z differences in the isotopic patterns observed are equal to one, single charged positive ions are formed. However, the fine structure also shows that individual peaks in the range m/z 750–920 (Fig. 6B) concern 2–3 different compounds rather than a single one.

After purification of HAs with γ -cyclodextrin according to the procedure described above some of the peaks in the mass spectrum are not observed (Fig. 6C). Mainly, the part around m/z 600 “disappeared” almost completely and the pattern of m/z 750–920 has changed so that the peaks are shifted to lower m/z values of $m/z \approx 50$. An example of a mass spectrum concerning γ -cyclodextrin soluble fraction is shown in Fig. 7. Because the m/z of γ -cyclodextrin $C_{48}H_{80}O_{40}$ is equal to 1296.42, the peaks 1361.94, 1395.28, 1431.5 and 1455.31 confirm the ionization of γ -cyclodextrin inclusion complexes containing in the CD cavity the compounds with M_r equal to 65.52, 98.86, 135.08 and 158.89 m/z . The first peak can be, for example, cyclopentadiene ($C_5H_6^+$ radical, m/z 66.0 theoretical, m/z observed 65.52). This compound is also found in coal tar and in distillates produced in carbonization of coal and so the observation of cyclopentadiene in HA is not surprising. Some of the peaks with low m/z values

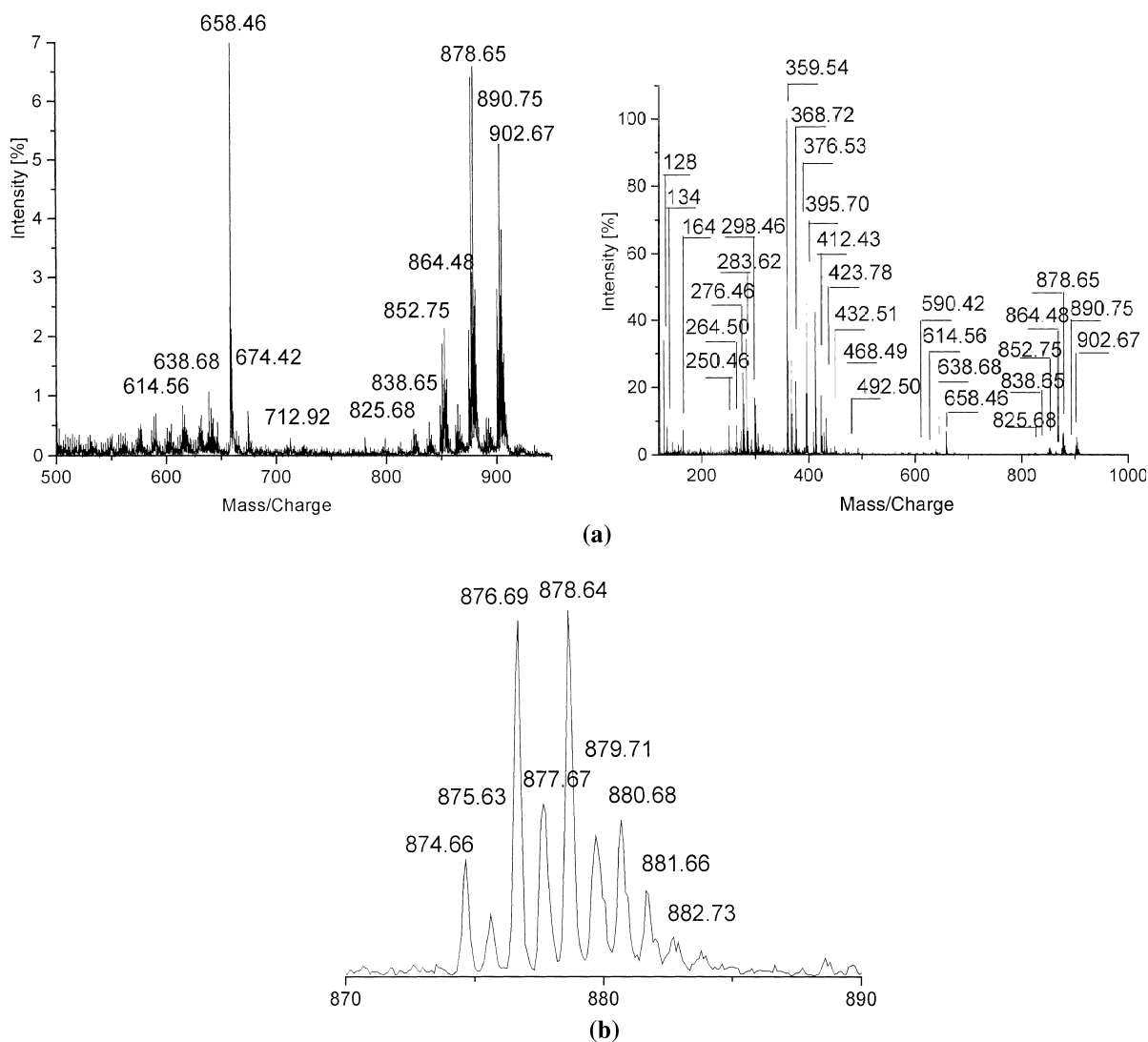


Fig. 6. (A) Mass spectra concerning soil IHSS standard humic acid. Linear positive mode, laser energy 70 units. (B) Mass spectra concerning soil IHSS standard humic acid in the range m/z 874–882. Linear positive mode, laser energy 70 units. (C) Mass spectra concerning soil IHSS standard humic acid purified with γ -cyclodextrin. Reflectron positive mode, laser energy 80 units.

(65.5, 135.1) are also observed in original (non-CD-purified) HAs.

Similar results were obtained for Antarctica samples. Examples of mass spectra concerning Antarctica humic acids are shown in Fig. 8A and B. It is evident, that some peaks around m/z 300 and 600 are also not observed in CD-purified material. The pattern (m/z 758–900) remains unchanged. An example of a mass spectrum concerning γ -cyclodextrin

soluble fraction is shown in Fig. 9. An almost identical pattern with several m/z peaks higher than γ -cyclodextrin is observed, with the same m/z values like those for the CD-soluble fraction of the soil IHSS standard. It can be concluded, that γ -cyclodextrin bounds low- M_r fractions of humic acids and that some of them can be ionized. This proves that in both, Antarctica HAs and in soil IHSS standard HA, there are several identical low- M_r compounds. In

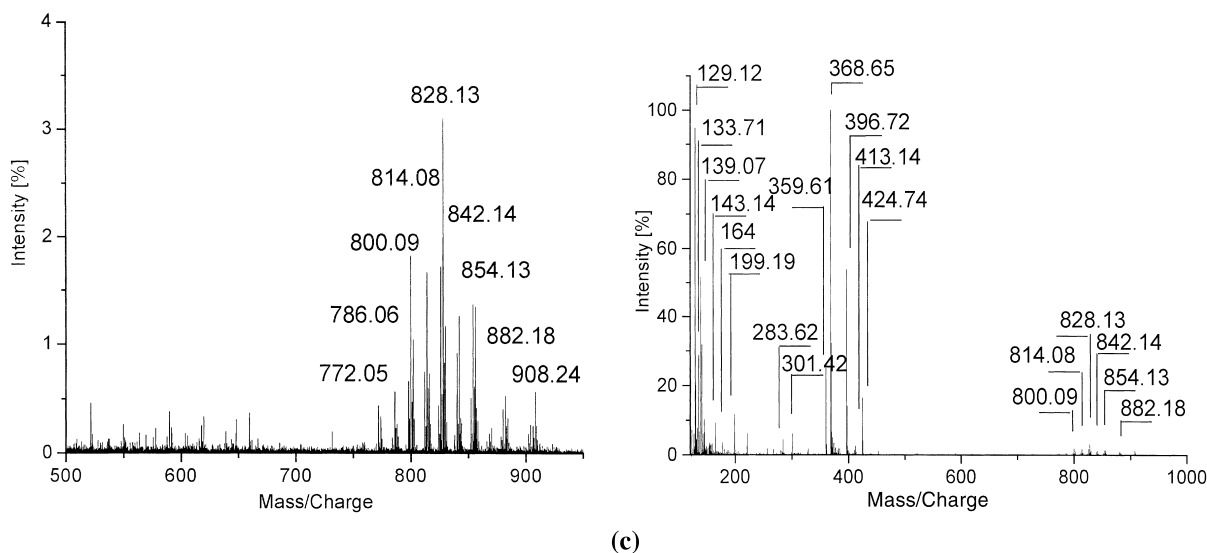


Fig. 6. (continued)

addition, higher- M_r compounds ($m/z \approx 750$ – 920) observed in Antarctica and in continental soil HAs are of very similar structure.

4. Conclusions

A new buffer, borate–Tris–EDTA modified with

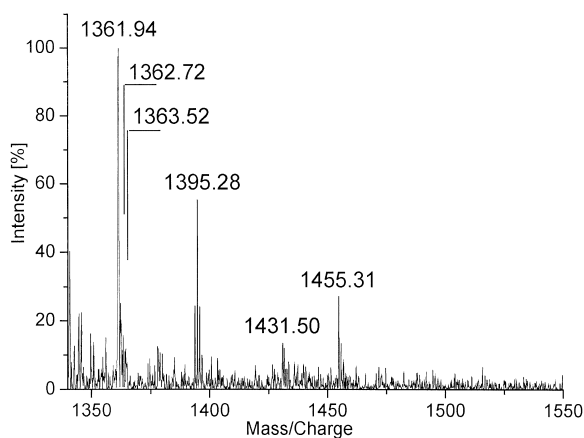


Fig. 7. Mass spectrum concerning γ -cyclodextrin extract of soil IHSS standard humic acid. Reflectron positive mode, laser energy 100 units.

0.03 M SDS+0.01 β -cyclodextrin mixture was found best for the characterization of HAs. An often observed unresolved “hump” of HAs was in this buffer separated into 5–7 peaks. We suppose that this is due to the partial distribution of some HA compounds from supramolecular HA aggregates to SDS micelles. In addition, several low- M_r HA constituents form inclusion complexes with CD and can be removed from HA supramolecule via precipitation under the presence of CD.

MS applied to analyze original, CD-purified HAs and CD-soluble fractions proved that HAs contain many low-molecular-mass simple compounds with M_r from ≈ 60 to a few hundred. When low- M_r fractions are removed from HA samples via formation of inclusion complexes with CD, then mass spectra of CD-purified HAs are simpler. Some of HA–CD inclusion compounds can be ionized and it was found that their M_r values are in the range m/z 65.0–158.4. The main part of HAs is presented by groups of compounds with $m/z \approx 770$ – 920 , which is the same not only for Antarctica and soil and peat from American continent but which is also observed in coal-derived humic acids. In addition, peaks with $m/z \approx 128$, 284, 360, 376, 658, 424, 590 and 658 observed in mass spectra in this work have also been detected in mass spectra of various humic acids from

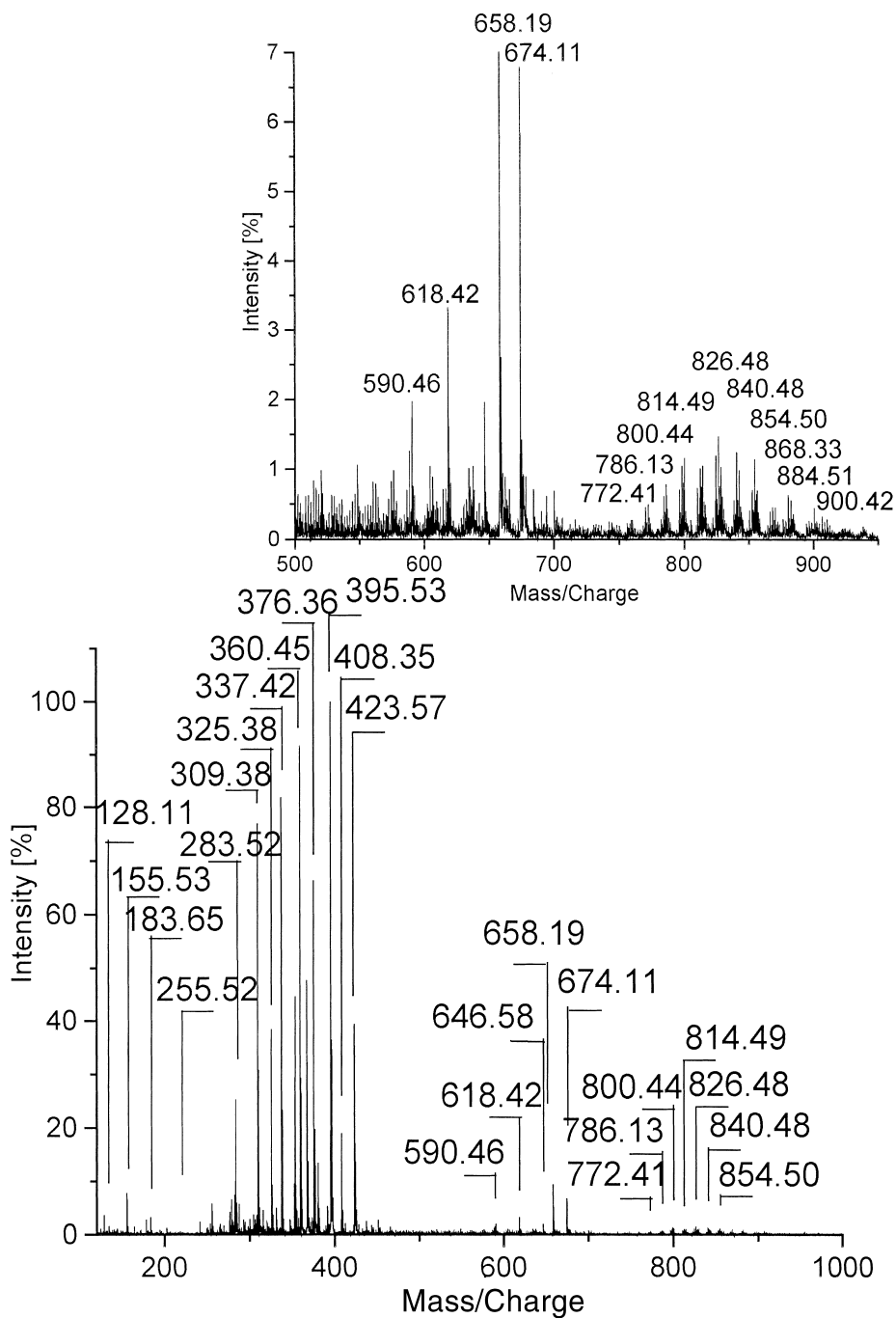


Fig. 8. (A) Mass spectra concerning Antarctica soil humic acid HA2-2002. Linear positive mode, laser energy 80 units. (B) Mass spectra concerning Antarctica soil humic acid HA2-2002 purified with γ -cyclodextrin. Linear positive mode, laser energy 100 units.

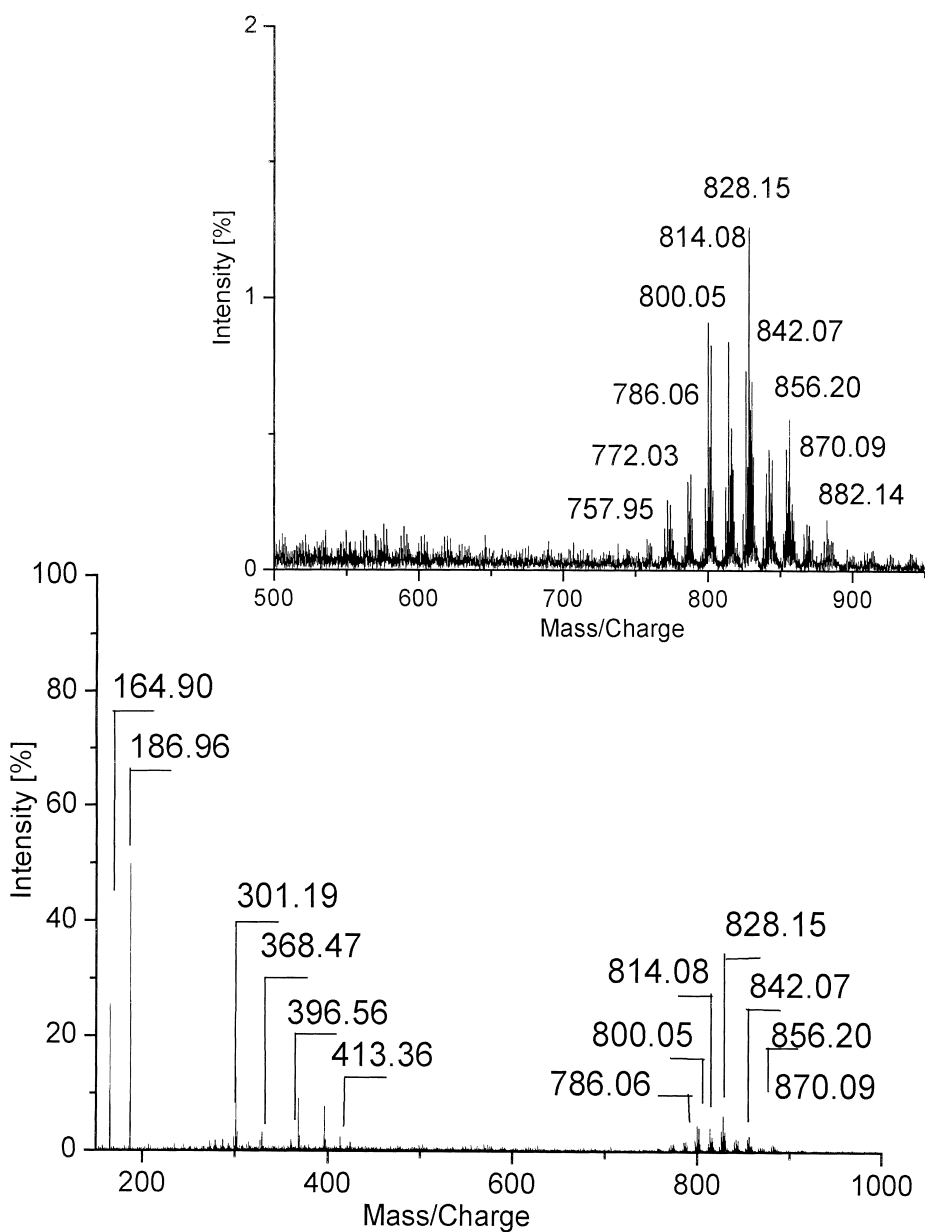


Fig. 8. (continued)

different continents and of different origin. In spite of a very different climate in Antarctica, it is shown that the humification process leads to humic organic matter containing the same compounds and/or similar structures.

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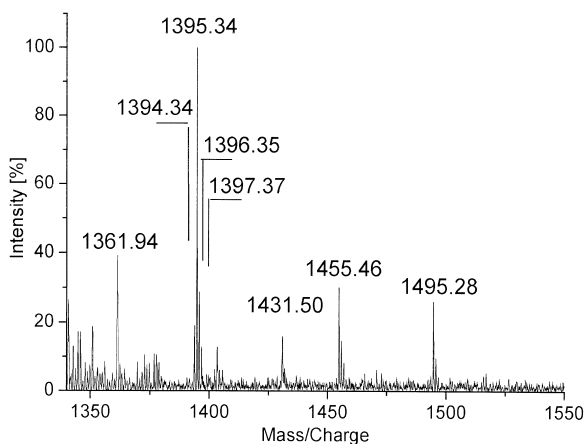


Fig. 9. Mass spectrum concerning γ -cyclodextrin extract of Antarctica humic acid HA2-2002. Reflectron positive mode, laser energy 92 units.

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