1 Introduction

Humic substances, the most abundantly occurring natural macromolecules, are extremely complex and heterogeneous compounds. The nonhomogeneity and the polydisperse nature of this material permit no complete picture of their composition and characteristics. For better understanding and characterization, careful and effective extraction and fractionation of these substances are considered indispensable. Several authors (DANNEBERG, 1973; KUTSCH, 1985; KUTSCH and DANNEBERG, 1985; DANNEBERG, 2001) demonstrated that extraction with a chelating resin and water is an exceedingly gentle method, combining a sufficient yield with a minimized chemical modification of the extracted material. For fractionation and characterization of humic substances chromatographic procedures are widely used. Controlled Pore Glass (CPG) is a suitable medium for fractionating humic substances according to molecular size because of its inert and non-adsorptive nature in contact with these substances (DANNEBERG, 1977; 2001); further, its rigid structure allows higher flow-rates and, thus, faster work than comparable organic gels (DANNEBERG, 1977; GERZABEK and ULLAH, 1989; TSUTSUKI and KAWATSUKA, 1979; NOVOTNY et al., 1999).

Zusammenfassung


Humic substances from three Austrian Cambisols were exhaustively extracted with a chelating resin and water and grossly fractionated into fulvic acids (FA) and brown humic acids (BHA). These, together with the unfractionated extract, were further investigated chromatographically using a column packed with Controlled Pore Glass (CPG) having a pore diameter of 16.7 nm. The effluents of the column were continuously analysed for the concentrations of both colored material and carbon, which were both recorded against partition coefficient ($K_d$). Specific extinctions per unit of C and at 400 nm were calculated in the course of the chromatograms and maximum values were taken as constants specific for ‘pure’ humic substances. Accompanying non humic substances were obtained by difference. Results revealed that Dystric Cambisol and Calcaric Cambisol had the typical distribution pattern of humic fractions, i.e. a domination of BHA; in Eutric Cambisol, however, grey humic acids (GHA, obtained by the difference extract – (BHA + FA)) were unusually high. The specific extinctions obtained for the ‘pure’ humic substances were in the expected range and comparable to literature values. FAs always showed a single elution peak with a $K_d$ of 0.7–0.75, BHA's had double peaks at $K_d$ values of 0.05 and 0.6, while GHA's had a couple of peaks between 0 and 0.55. The color quotients of the different ‘pure’ humic fractions in the studied soils were shown to have usual ratios, i.e. inversely related with the molecular size of the humic substances. Non humic substances were, in general, characterized by different fractions as well low, medium and high in molecular size. The ratio of ‘pure’ humic substances to non humic substances was, as expected, approximately 2:1.

Key words: Cambisols, humic system, chromatography, Controlled Pore Glass, ‘pure’ humic substances, non humic substances.

2 Materials and methods

2.1 Soils

Three subtypes of Cambisols were sampled for a depth of 0–20 cm in the beginning of spring, 1998. These three locations were all devoted to agricultural practices and situated in Lower Austria. The collected soil samples were air-dried and sieved (2 mm). Routine soil analysis was carried out with these samples following standard methods. The soil subtypes, the location of sampling and some of their salient features are presented in table 1.

2.2 Extraction and freeze dry preparation of extracts

The soils were exhaustively extracted for four consecutive days with a chelating resin (Chelex-100, Na-form) and water to get maximum possible yields (DANNEBERG, 1981; 2001). Extracted materials from each day were separated by high speed centrifugation, collected and combined in one container, deep-frozen, freeze dried and stored in a refrigerator until further use.

2.3 Gross fractionation and optical analysis

The freeze dried extracts were dissolved in water, made to volume and aliquots were taken for gross fractionation into fulvic acids (FA) and brown humic acids (BHA) according to DANNEBERG (2001). These, together with aliquots of the unfractionated extract, were freeze dried and kept under cooling for chromatography. Other aliquots, treated in the same way, were used for optical analysis; extinctions at
wavelengths of 400 and 600 nm were recorded in buffered solution (pH = 10.0) with a spectrophotometer. Grey humic acids (GHA) were not directly prepared since they cannot be readily redissolved once they are precipitated; they were obtained by difference \[\text{GHA} = \text{extract} - (\text{FA} + \text{BHA})\].

### 2.4 Chromatographic experiment

Column set up: A glass column of 16 mm internal diameter was packed with Cotrolled Pore Glass (CPG, obtained from CPG Inc., USA) with a pore diameter of 16.7 nm and a grain size of 120–200 mesh to obtain a chromatographic column about 600 mm in length. The column was fitted with a flow adapter. A compact and even packing of the CPG is essential. The column was then equilibrated with the elution solution, containing 0.02 moles Na$_2$B$_4$O$_7$ and 0.05 moles NaCl per litre in CO$_2$-free water. Calibration parameters of the column were determined using a test-solution of 1 % benzyl alcohol (to determine $V_t$) and 0.5 % Blue Dextran 2000 (obtained from Pharmacia, Sweden; to determine $V_0$) in elution solution. The column outlet was first passed through the UV-monitor to have a control of the proper run. It was then either introduced into the carbon-monitor or it was collected in a fraction collector, the fractions being subsequently analysed with a spectrophotometer. Thus, for every sample two runs were carried out, one for optical analysis and one for carbon determination.

Carbonate estimation: To estimate the amount of carbonates which were co-extracted from soils containing lime, separate runs were carried out with Na$_2$CO$_3$-solution of different concentrations, keeping all other experimental set up the same. The closest of these chromatographically obtained and integrated values was then subtracted from the integrated value of the total extract to correct for its carbonate content (DANNEBERG and ULLAH, 1982).

Calculation: The direct results of chromatographic experiments were recorder-plottings of either extinction or carbon concentration vs. elution volume. Since elution volume depends on the dimensions of column and tubing connections it is not suitable for comparison; therefore, it is

<table>
<thead>
<tr>
<th>No.</th>
<th>Soil types (FAO) /names and location</th>
<th>Sand %</th>
<th>Silt %</th>
<th>Clay %</th>
<th>Soil pH</th>
<th>Lime %</th>
<th>Org. Matter %</th>
<th>N-total %</th>
<th>CEC Meq/100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dystric Cambisol Felsbraunerde, non-calc. Langau (KB 36)</td>
<td>44</td>
<td>45</td>
<td>11</td>
<td>6.1</td>
<td>0</td>
<td>1.50</td>
<td>0.12</td>
<td>6.9</td>
</tr>
<tr>
<td>2</td>
<td>Eutric Cambisol Lockersediment – Braunerde, non-calc. Zistersdorf (KB 101)</td>
<td>6.0</td>
<td>66</td>
<td>28</td>
<td>7.0</td>
<td>0</td>
<td>2.40</td>
<td>0.17</td>
<td>21.4</td>
</tr>
<tr>
<td>3</td>
<td>Calcaric Cambisol Lockersediment – Braunerde, calc. Zistersdorf (KB 101)</td>
<td>9.0</td>
<td>68</td>
<td>23</td>
<td>7.5</td>
<td>4.1</td>
<td>2.80</td>
<td>0.16</td>
<td>21.5</td>
</tr>
</tbody>
</table>
converted to the partition coefficient, $K_d$. This is calculated using the following formula:

$$K_d = \frac{Ve - Vo}{Vt - Ve}$$

Where, $Ve = \text{Elution volume}$,

$Vo = \text{Void volume}$ and

$Vt = \text{Total volume}$

To arrive at both extinction values and carbon concentrations corresponding to equal $K_d$-values, results were interpolated for given and round values of $K_d$. From this set of data, specific extinctions were calculated for unit of C as a measure of “purity” of humic substances. The maximum value characterizes the purest fraction and was used for the computation of “pure” humic substances, i.e. to convert extinction values to corresponding concentrations of carbon using Lambert–Beer’s Law (SCHNITZER, 1971; DANNEBERG, 1981; 2001).

Non humic substances were simply obtained by differences, i.e.,

$$C_{\text{non humic substances}} = C_{\text{total}} - C_{\text{"pure" humic substances}}$$

All calculations as well as graphic presentations of results were carried out with EXCEL.

3 Results and discussion

Before the chromatographic experiments, the total extracts of the soils under study and their gross humic fractions were optically analysed at 400 nm to get a general view of the distribution pattern of their humic systems. The results of these determinations are presented in terms of optical density per gram of soil (OD/g) in table 2 and their percentages in figure 1.

Results demonstrate that, in terms of humic fraction distribution pattern, the Dystric and the Calcaric Cambisol are similar to each other, while the Eutric Cambisol differs considerably.

Brown humic acids were the dominant fraction (53%) in Dystric Cambisol and the contents of brown and grey humic acids were nearly equal in Calcaric Cambisol. Thus, the results of the latter two soils were in agreement with the “Braunerde” humic systems as shown in the literature (DANNEBERG and SCHMIDT, 1978; KUTSCH and DANNEBERG, 1985). In the Eutric Cambisol, on the other hand, the content of colored matter in the total extract was appreciably high, the lion’s share of which was constituted of high molecular grey humic acids (77%), which seems quite unusual and resembles closely with a Chernozem humic system (DANNEBERG and ULLAH, 1982). The sampling area of this arable soil is dry and encircled by black soils of Chernozem type. Thus, the unexpected high concentration of grey humic acids might be indicative to possible transitional developments between Cambisol and Chernozem. However, this tendency of the humic system certainly needs further investigations.

The chromatograms of the different humic fractions of the soils under study are presented in the figures 2, 3 and 4.

Table 2: Contents of gross humic fractions in the soils under study

<table>
<thead>
<tr>
<th>No.</th>
<th>Soils</th>
<th>Total extracts</th>
<th>Fulvic Acids</th>
<th>Brown Humic Acids</th>
<th>Grey Humic Acids</th>
<th>HA/FA ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dystric Cambisol</td>
<td>2.16</td>
<td>0.49</td>
<td>1.15</td>
<td>0.52</td>
<td>3.41</td>
</tr>
<tr>
<td>2</td>
<td>Eutric Cambisol</td>
<td>9.33</td>
<td>0.54</td>
<td>1.59</td>
<td>7.20</td>
<td>16.28</td>
</tr>
<tr>
<td>3</td>
<td>Calcaric Cambisol</td>
<td>2.42</td>
<td>0.54</td>
<td>0.91</td>
<td>0.97</td>
<td>3.48</td>
</tr>
</tbody>
</table>

![Figure 1: Percent distribution of different gross humic fractions in the soils under study](image1.png)

Abbildung 1: Verteilung der Grobfraktionen der Huminstoffe in den untersuchten Böden
Among the chromatograms of different humic fractions, fulvic acids present a sharp, clear and uniform single peak of more upright nature at a K_d-range of 0.70–0.75, which signifies that fulvic acids are eluted properly at the end of the elution diagram and are, thus, of lower molecular size than the other humic fractions (Fig. 2a, 3a and 4a).

The chromatograms of brown humic acids exhibit two peaks at different K_d-positions, a tiny macromolecular part at a K_d near zero and a major peak at K_d of 0.60 in all instances (Fig. 2b, 3b and 4b).

The elution curves of grey humic acids are also evidently characterized by distinct double peaks, separating the fractions into two. The macromolecular fractions are obtained on the exclusion side of the diagrams at K_d of zero and the other mixed fractions at K_d-ranges of 0.50–0.55 (Fig. 2c, 3c and 4c).

A direct comparison of these findings with literature values is difficult, because the chromatographic material used is not identical. However, the shape and elution patterns of the different fractions are in good agreement and K_d-values are close to literature values (DANNEBERG and SCHMIDT, 1978; DANNEBERG and ULLAH, 1982; KUTSCH and DANNEBERG, 1985; MOHAMAD et al., 1993; NOVOTNY et al., 1999).

Table 3 summarizes the chromatographic results by integration of the chromatographic peaks and presentation of all the fractions obtained in terms of mg carbon per gram of soil and, to arrive at a complete picture, as percent of extracted organic matter.

The specific extinctions of the “pure” humic fractions are presented in table 4.

In a German Luvisol, KUTSCH and DANNEBERG (1981) found specific extinctions of 39.4, 155.8 and 349.6 for FA, BHA and GHA, respectively. These values compare quite well with the results obtained here for all the fulvic acids and for the GHA’s of the Eutric Cambisol. The Dystric as well as the Calcaric Cambisol, however, show higher values for the BHA’s and lower for the GHA’s. The corresponding values of the two soils compare well to each other. All values are significantly smaller than the corresponding values reported for a Chernozem (DANNEBERG and ULLAH, 1982).
Figure 3 (a–d): Chromatograms of different humic fractions of Eutric Cambisol
Abbildung 3 (a–d): Chromatogramme der Huminstoff-Grobfraktionen aus der kalkfreien Lockersedimentbraunerde

Table 3: Chromatographic results of different humic fractions of the soils under investigation
Tabelle 3: Integrierte chromatographische Fraktionen in den untersuchten Böden

<table>
<thead>
<tr>
<th>Gross fractions</th>
<th>Dystric Cambisol</th>
<th>Eutric Cambisol</th>
<th>Calcaric Cambisol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>'mg' C/g</td>
<td>%</td>
<td>'mg' C/g</td>
</tr>
<tr>
<td>Sum HS 'Pure'</td>
<td>1.89</td>
<td>65.15</td>
<td>3.12</td>
</tr>
<tr>
<td>'Pure' FA</td>
<td>0.33</td>
<td>11.32</td>
<td>0.30</td>
</tr>
<tr>
<td>'Pure' BHA</td>
<td>0.99</td>
<td>34.30</td>
<td>0.51</td>
</tr>
<tr>
<td>'Pure' GHA</td>
<td>0.57</td>
<td>19.53</td>
<td>2.31</td>
</tr>
<tr>
<td>Sum NHS</td>
<td>1.01</td>
<td>34.85</td>
<td>1.60</td>
</tr>
<tr>
<td>FA I</td>
<td>0.02</td>
<td>0.66</td>
<td>0.02</td>
</tr>
<tr>
<td>FA II</td>
<td>0.05</td>
<td>1.83</td>
<td>0.05</td>
</tr>
<tr>
<td>FA III</td>
<td>0.04</td>
<td>1.41</td>
<td>–</td>
</tr>
<tr>
<td>BHA I</td>
<td>0.21</td>
<td>7.28</td>
<td>0.10</td>
</tr>
<tr>
<td>BHA II</td>
<td>0.21</td>
<td>7.11</td>
<td>0.19</td>
</tr>
<tr>
<td>BHA III</td>
<td>0.03</td>
<td>1.17</td>
<td>0.03</td>
</tr>
<tr>
<td>GHA I</td>
<td>0.09</td>
<td>3.17</td>
<td>0.29</td>
</tr>
<tr>
<td>GHA II</td>
<td>0.08</td>
<td>2.66</td>
<td>0.12</td>
</tr>
<tr>
<td>GHA III</td>
<td>0.28</td>
<td>9.56</td>
<td>0.81</td>
</tr>
<tr>
<td>Total</td>
<td>2.90</td>
<td>100.00</td>
<td>4.72</td>
</tr>
<tr>
<td>Lime</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Percent value of total lime contents, HS: Humic substances, NHS: Non humic substances.
The colour quotients \( \left( \frac{E_{400}}{E_{600}} \right) \) of the different "pure" fractions, i.e. recorded at the positions with the maximum of specific extinction in the chromatogram, are presented in table 5. Colour quotients of high molecular GHA's ranged between 3.59 and 4.05, those of medium sized BHA's between 5.06 and 5.33, while low molecular FA's show values from 6.69 to 7.82. These results are well corroborated with other findings (DANNEBERG and SCHMIDT, 1978; MARTIN et al., 1998; NOVOTNY et al., 1999; TRUBETSKOJ et al., 1999).

Comparing the colour quotients of the three soils especially that of the GHA in Eutric Cambisol is somewhat low, which indicates greater association of molecules with higher levels of condensations of aromatic rings in this fraction. This finding underlines that the humic system of this soil is intermediate to Chernozem humic systems.
The accompanying non humic substances in the three gross fractions, as given in table 3, were found to be present in variable amounts and, in most cases, were characterized by two to three distinguishable fractions of different molecular size. Of them, the first peaks at the exclusion part of the chromatogram, represent materials of high molecular size, the second peaks are of intermediate size and the third peaks at the tail of the elution curves account for low molecular materials.

It is interesting to notice some similarities in the Kd-position of different non humic fractions among the studied gross humic fractions: As can be seen from table 3, BHA I and GHA I at Kd = 0.05 in Eutric Cambisol, FA I and BHA I at Kd = 0.0 and FA III and BHA III at Kd = 0.85 in Dystric Cambisol as well as in Calcaric Cambisol show equal or similar positions. This might indicate similarities not only in molecular size but also in the chemical nature of this non humic fractions; however, this requires further investigations.

The sum of non humic fractions in the soils under study makes up a very constant part of 33 %, while the "pure" humic substances make up 66 %. This is in agreement with older findings (DANNEBERG and ULLAH, 1982; KUTSCH and DANNEBERG, 1985).

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References


Address of authors

Univ. Assoc. Prof. Dr. Abdus Salam M. Mohiuddin, Department of Soil, Water and Environment, University of Dhaka, Dhaka-1000, Bangladesh
E-Mail: asmm@mailcity.com

Univ. Doz. Dipl.-Ing. Dr. Otto H. Danneberg, Johann Hörbigergasse 18, 1230 Wien, Austria

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