Characterization of humic substances from Austrian cambisols by chromatography on CPG

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Charakterisierung von Huminstoffsystemen österreichischer Braunerden durch Chromatographie an porenkontrolliertem Glas

1 Introduction

Humic substances, the most abundantly occuring 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

Die Huminstoffe von drei österreichischen Braunerden wurden mit einem komplexbildenden Ionentauscher und Wasser erschöpfend extrahiert und die Extrakte grob in Fulvosäuren (FA) und Braunhuminsäuren (BHA) fraktioniert. Diese Grobfraktionen und der unfraktionierte Extrakt wurden durch Chromatographie an einer Säule mit porenkontrolliertem Glas (CPG) von 16,7 nm Porendurchmesser weiter untersucht. Das abfließende Säuleneluat analysierten wir kontinuierlich auf seine Konzentration von gefärbten Substanzen und von Kohlenstoff, beide Parameter wurden als Chromatogramm dem Verteilungskoeffizienten (K_d) gegenübergestellt. Daraus berechneten wir die auf die Einheit von Kohlenstoff bezogene spezifische Extinktion bei 400 nm im Verlauf der Chromatogramme und verwendeten das Maximum dieser Reihe als Stoffkonstante der „reinen" Huminstoffe; die begleitenden Nichthuminstoffe errechneten wir als Differenz.

Die Ergebnisse zeigten sowohl für die Felsbraunerde als auch für die kalkhaltige Lockersedimentbraunerde die erwartete Verteilung der Grobfraktionen mit einer deutlichen Dominanz der BHAs. Dagegen war in der kalkfreien Lockersedimentbraunerde ein unerwartet hoher Anteil der Grauhuminsäuren [GHA, erhalten als Extrakt – (FA + BHA)] festzustellen. Die erhaltenen spezifischen Extinktionen der „reinen" Huminstoffe lagen durchwegs im erwarteten Bereich und waren mit Werten der Literatur gut vergleichbar. Die Fulvosäuren zeigten immer einen Elutionspeak mit einem K_d-Wert von 0,7 bis 0,75. Die Braunhuminsäuren hatten Doppelpeaks mit K_d-Werten von 0,05 und 0,6; die Grauhuminsäuren dagegen zeigten mehrere Peaks mit K_d-Werten zwischen 0 und 0,55. Die Farbquotienten der „reinen" Huminstoff-Fraktionen zeigten die üblichen Werte, sie waren umso größer, je kleiner die Molekülgrößen waren. Die Nichthuminstoffe ergaben mehrere Peaks, es ließen sich hohe, mittlere und niedrige Molekülgrößen unterscheiden. Das Verhältnis der „reinen" Huminstoffe zu begleitenden Nichthuminstoffen war, wie erwartet, etwa 2:1.

Schlagworte: Braunerden, Chromotographie, porenkontrolliertes Glas, „reine" Huminstoffe, Huminstoffsystem, Nichthuminstoffe.

Summary

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 partitition 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.

Non humic substances such as carbohydrates, proteins, peptides, amino acids, fats, waxes, low molecular organic acids etc. always accompany humic materials as coextracted non-coloured organic impurities because a selective extraction is not possible. Most of these non humic substances are readily attacked by microorganisms and usually have only a short live span in soil (SCHNITZER, 1991). Consequently, the calculation of the ratio of humic to non humic substances gives a hint on the humus dynamics in different soils (DANNEBERG, 1981; 2001).

Cambisol is the most abundant soil in Austrian landscape. Relatively few investgations were made concerning the humic substances of this type of soil. So, the present paper aims to give some information on these humic systems, which might contribute to a better understanding of them.

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

Table 1: Some characteristic physical and chemical features of the soils under study
Tabelle 1: Einige physikalische und chemische Eigenschaften der untersuchten Böden

No.	Soil types (FAO) /names and location	Sand %	Silt %	Clay %	Soil pH $CaCl_2$	Lime %	Org. Matter %	N-total %	CEC Meq/100 g
1	**Dystric Cambisol** Felsbraunerde, non-calc. Langau (KB 36)	44	45	11	6.1	0	1.50	0.12	6.9
2	**Eutric Cambisol** Lockersediment – Braunerde, non-calc. Zistersdorf (KB 101)	6.0	66	28	7.0	0	2.40	0.17	21.4
3	**Calcaric Cambisol** Lockersediment – Braunerde, calc. Zistersdorf (KB 101)	9.0	68	23	7.5	4.1	2.80	0.16	21.5

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 [GHA = extract – (FA + 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 $Na_2B_4O_7$ 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 connected to a continuous flow UV-monitor and, in turn, to a fraction collector or a C-monitor. The UV-monitor and the C-monitor both were connected to a two-channel plotter.

C-monitor set up: An instrument designed for the determination of Dissolved Organic Carbon (DOC) in natural water (TOCOR 2, MAIHAK) was used as a C-monitor. The instrument was calibrated with standard solutions containing known amounts of potassium-hydrogen-phthalate, dissolved in CO_2-free water as a source of organic carbon.

Chromatographic analysis: The whole experiment was carried out according to DANNEBERG (1981; 2001). Since soil humic substances contain a heterogeneous mixture of substances with different molecular sizes and shapes and for which each group of compounds may exhibit a different relationship between log MW and partition coefficient (K_d), a calibration of the column on the basis of molecular weight has not been tried (SADAO and BARTH, 1999). Freeze dried aliquots of unfractionated extract, FA and BHA were dissolved in a proper volume of elution solution. 2 ml of these sample solutions were introduced separately onto the column through a sample loop and eluted with elution solution using a flow rate of approximately 1 ml/min. The column effluent 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_2CO_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

converted to the partitition coefficient, K_d. This is calculated using the following formula:

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

Where, Ve = Elutionvolume
Vo = Void volume and
Vt = 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.,

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

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
Tabelle 2: Gehalte an Grobfraktionen der Huminstoffe in den untersuchten Böden

No.	Soils	Total extracts	Fulvic Acids	Brown Humic Acids	Grey Humic Acids	HA/FA ratio
1	**Dystric Cambisol**	2.16	0.49	1.15	0.52	3.41
2	**Eutric Cambisol**	9.33	0.54	1.59	7.20	16.28
3	**Calcaric Cambisol**	2.42	0.54	0.91	0.97	3.48

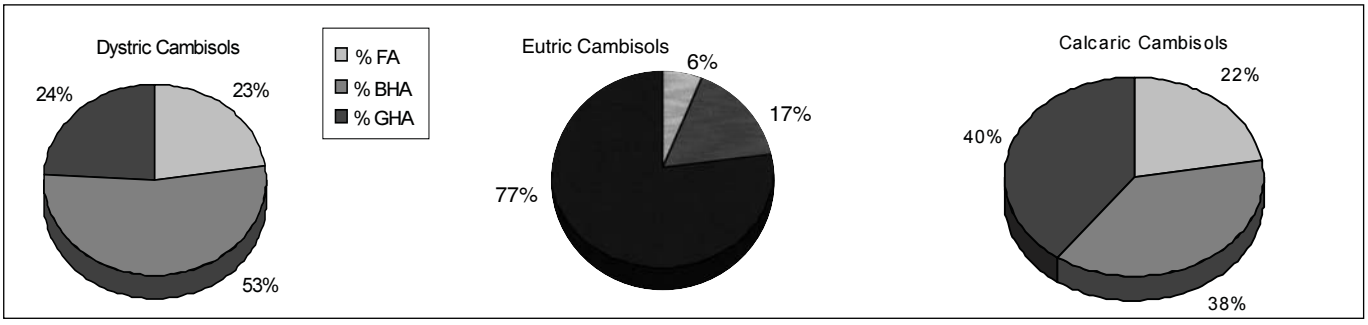

Figure 1: Percent distribution of different gross humic fractions in the soils under study
Abbildung 1: Verteilung der Grobfraktionen der Huminstoffe in den untersuchten Böden

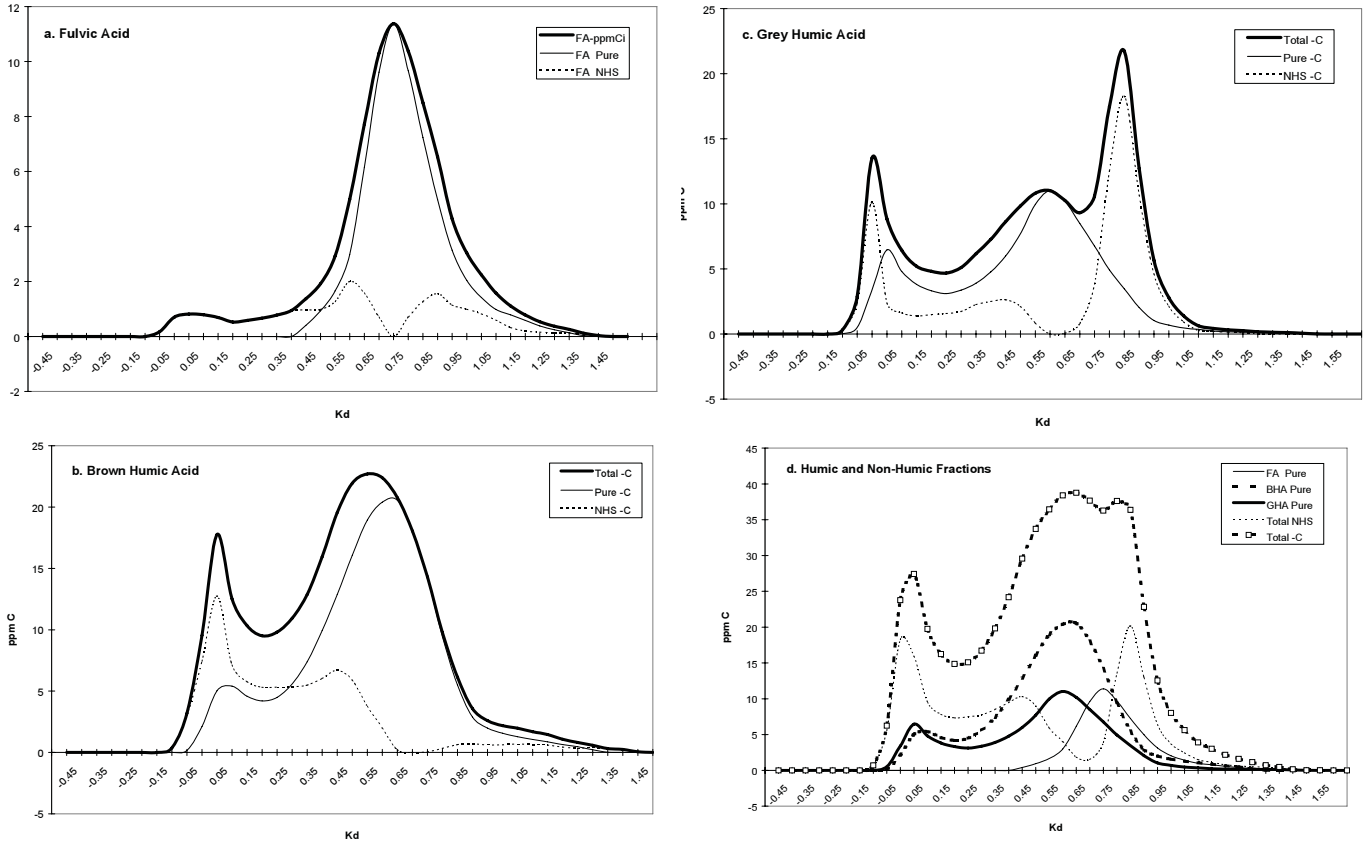

Figure 2 (a–d): Chromatograms of different humic fractions of Dystric Cambisol
Abbildung 2 (a–d): Chromatogramme der Huminstoff-Grobfraktionen aus der Felsbraunerde

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).

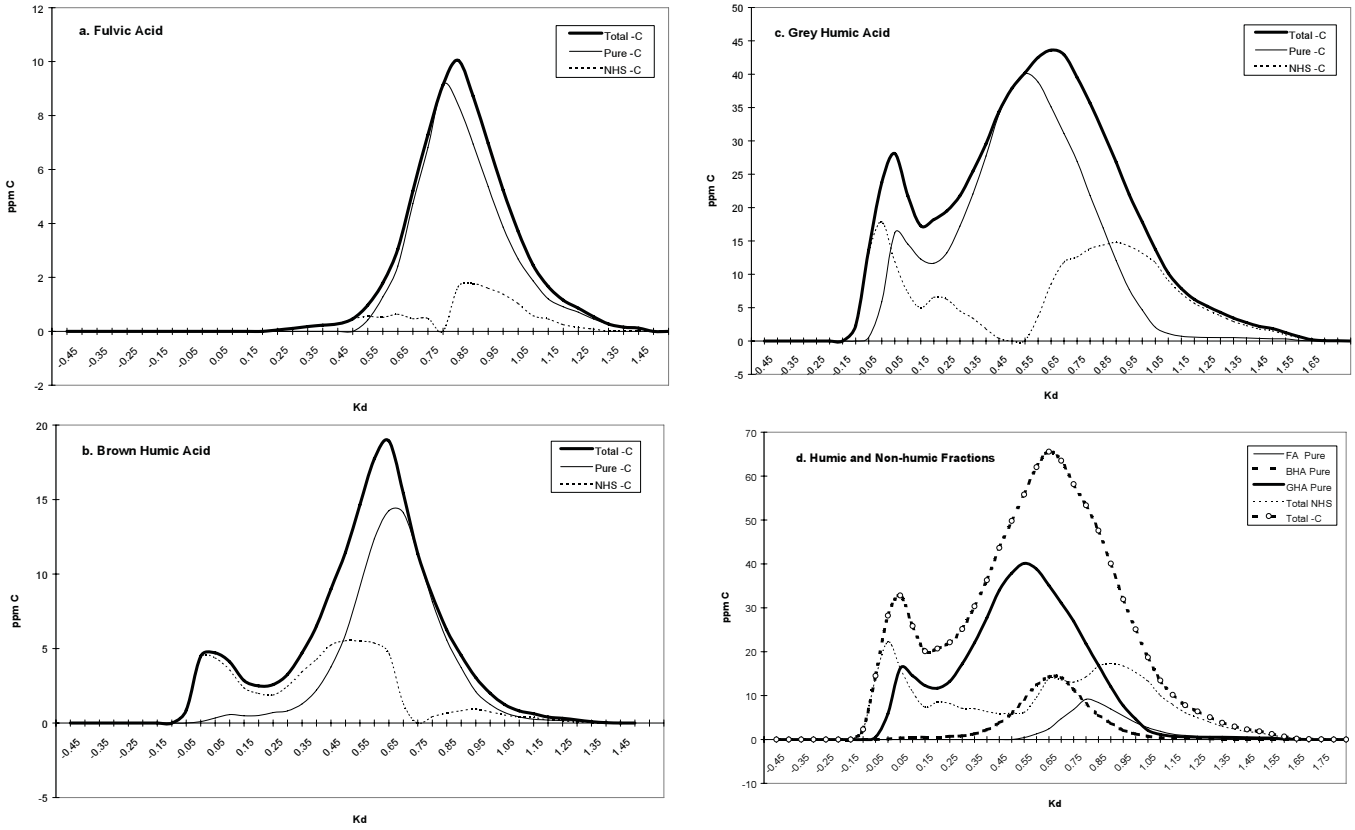

Figure 3 (a–d): Chromatograms of different humic fractions of Eutric Cambisol
Abbildung 3 (a–d): Chromatogramme der Huminstoff-Grobfraktionen aus der kalkfreien Lockersedimentbraunerde

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

Gross fractions	Dystric Cambisol		Eutric Cambisol		Calcaric Cambisol	
	'mg' C/g	%	'mg' C/g	%	'mg' C/g	%
Sum HS 'Pure'	**1.89**	**65.15**	**3.12**	**66.09**	**2.40**	**65.58**
'Pure' FA	0.33	11.32	0.30	6.25	0.54	14.68
'Pure' BHA	0.99	34.30	0.51	10.83	0.85	23.32
'Pure' GHA	0.57	19.53	2.31	49.00	1.01	27.58
Sum NHS	**1.01**	**34.85**	**1.60**	**33.87**	**1.26**	**34.42**
FA I	0.02	0.66	0.02	0.40	0.03	0.87
FA II	0.05	1.83	0.05	0.97	0.09	2.54
FA III	0.04	1.41	–	–	0.05	1.31
BHA I	0.21	7.28	0.10	2.08	0.13	3.50
BHA II	0.21	7.11	0.19	4.07	0.13	3.64
BHA III	0.03	1.17	0.03	0.57	0.10	2.71
GHA I	0.09	3.17	0.29	6.17	0.33	8.94
GHA II	0.08	2.66	0.12	2.48	0.40	10.91
GHA III	0.28	9.56	0.81	17.13	–	–
Total	**2.90**	**100.00**	**4.72**	**99.96**	**3.65**	**100.00**
Lime	–	–	–	–	3.15	7.67*

* Percent value of total lime contents, HS: Humic substances, NHS: Non humic substances.

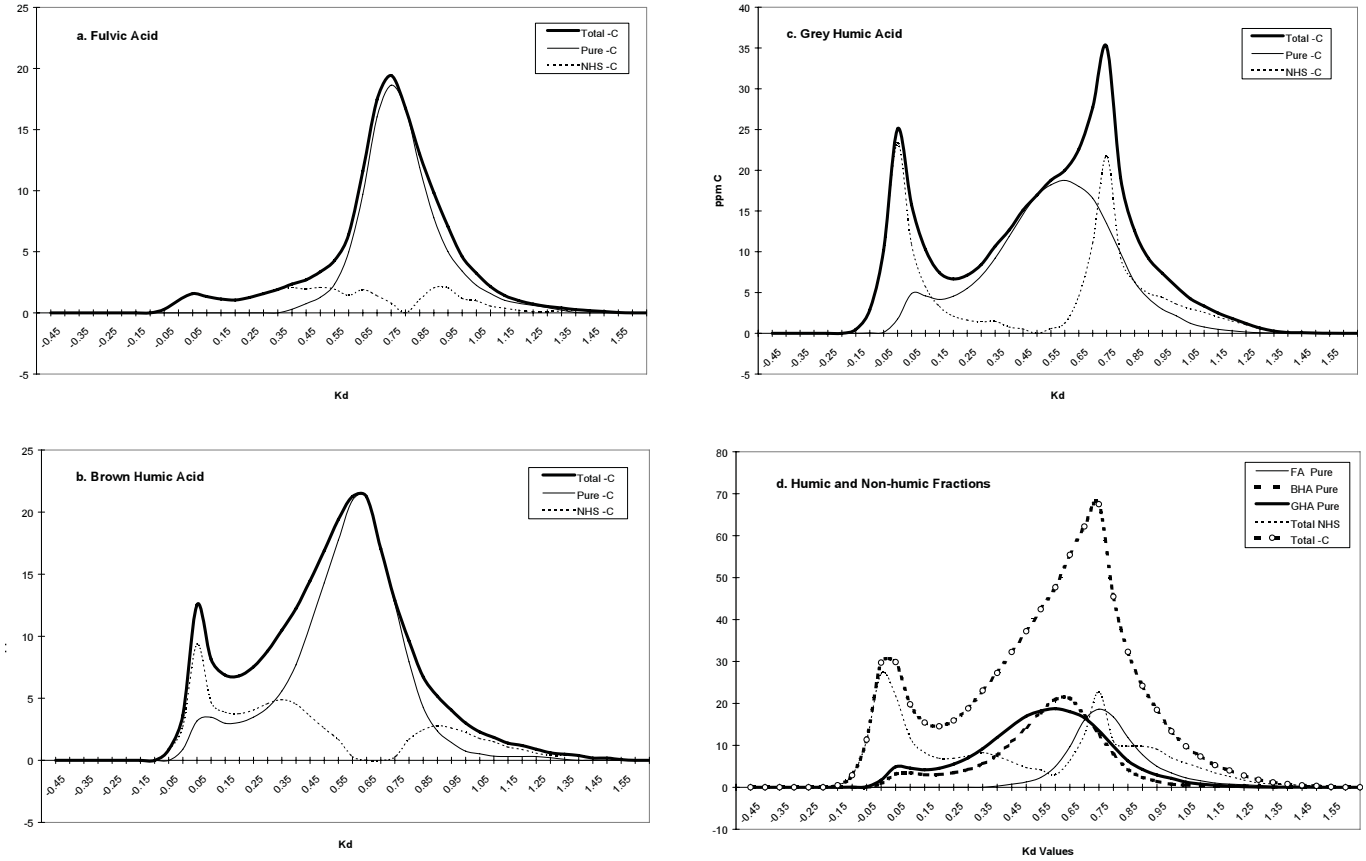

Figure 4 (a–d): Chromatograms of different humic fractions of Calcaric Cambisol
Abbildung 4 (a–d): Chromatogramme der Huminstoff-Grobfraktionen aus der kalkhaltigen Lockersedimentbraunerde

Table 4: Specific extinctions of the different "pure" humic fractions of the soils under study
Tabelle 4: Spezifische Extinktionen der „reinen" Huminstofffraktionen aus den untersuchten Böden

No.	Soils	'Pure' Fulvic Acids	'Pure' Brown Humic Acids	'Pure' Grey Humic Acids
1	**Dystric Cambisol**	33.2	206.7	217.8
2	**Eutric Cambisol**	30.7	337.9	373.2
3	**Calcaric Cambisol**	41.1	209,7	231.5

The colour quotients (E_{400}/E_{600}) 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).

Table 5: Colour quotients of the different "pure" humic fractions of the soils under study as obtained from chromatographic experiments
Tabelle 5: Farbquotienten der "reinen" Huminstofffraktionen, erhalten aus den chromatographischen Experimenten

Soils	'Pure' FA (peak)	'Pure' BHA (2^{nd} peak)	'Pure' GHA (2^{nd} peak)
Dystric Cambisol	6.85	5.06	3.86
Eutric Cambisol	6.69	5.33	3.59
Calcaric Cambisol	7.82	5.17	4.05

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 K_d-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 K_d = 0.05 in Eutric Cambisol, FA I and BHA I at K_d = 0.0 and FA III and BHA III at K_d = 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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