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Literature review on:

Soil Organic Matter Characterization

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Summary

This paper has been written to give the reader a review of extraction, purification, fractionation and characterization techniques available for soil organic matter characterization. The review is divided into chapters on each of the techniques mentioned.

As far as extraction techniques are concerned there are two categories: bulk extractions and sequential extraction methods. A humin extraction method was also found.

Purification methods are numerous and it seems that purification is always necessary to make functional groups accessible to the reagents used in subsequent characterization steps.

Fractionation techniques are all more or less based on the chromatography principle and can be divided into the following categories: fractionation based on differences in molecular size, chemical properties, and mobility in electrical fields.

In the field of characterization techniques two divisions can be made: spectroscopical methods (E_4/E_6 ratio, PYGCMS, IR, NMR etc.) and chemical methods (functional group titration).

In the end a chapter on applications in the field of interactions of pesticides with soil organic matter has been written.

From the literature it appeared that there is not a generally accepted standard characterization method.

Samenvatting

Dit rapport is bedoeld om de lezer een overzicht te geven van extractie-, zuiverings-, fractionerings- en karakteriseringstechnieken die gebruikt worden in de karakterisering van organisch materiaal in de bodem. Het rapport is onderverdeeld in hoofdstukken waarin ieder van de hierboven genoemde technieken wordt behandeld.

De extractie technieken kunnen worden onderverdeeld in twee categorieën: bulk- en sequentiële extracties. Er is ook een extractie methode voor humine gevonden.

Fractioneringstechnieken zijn alle min of meer gebaseerd op het principe van chromatografie. Deze methoden kunnen worden onderverdeeld in de volgende categorieën: fractionering gebaseerd op verschillen in molecuul grootte, chemische eigenschappen en mobiliteit in electrische velden.

Op het gebied van karakteriseringsmethoden kunnen er twee onderverdelingen gemaakt worden: spectroscopische methoden (E_4/E_6 ratio, PYGCMS, IR, NMR etc.) en chemische methoden (titraties van functionele groepen).

Tenslotte is er een hoofdstuk geschreven over toepassingen op het gebied van interacties van pesticiden met organisch materiaal in de bodem.

Uit de literatuur bleek verder dat er geen algemeen geaccepteerde standaard karakteriserings methode is.

1. Introduction

For several projects on soil and groundwater research it is necessary to obtain more knowledge concerning soil organic matter.
For prediction of the behaviour of compounds in soil and groundwater the organic matter in the soil is often supposed to be built up from homogeneous material with standard sorption properties.

Sorption properties for organic compounds as well as metals are often described as a function of the organic matter content; environmental reference values are in the Netherlands differentiated to the content of soil organic matter [1].
However, in reality soil organic matter is a complex material (Fig. 1) which is built up out of several different types of organic species with different characteristic properties. For example, organic matter in peat has complete different characteristics than organic matter in a podzol. More knowledge concerning the characteristics of organic matter fractions however might be necessary for a better understanding and description, and to give better tools to solve environmental problems like:
- the sorption of organic compounds like e.g. pesticides and polycyclic aromatic hydrocarbons
- the bioavailibilty of chemicals in the soil
- the sorption of metal ions in the soil

The aim of this study is:

A. to draw up an inventory of the different techniques available for fractionation and characterization of soil organic matter

B. to propose a standard method for characterization of the soil organic matter in the soil in order to standardize and apply it for environmental soil research.

The composition of a soil can roughly be described as follows:

- mineral matter
  a) silicates, e.g. clay minerals (smectite, illite, kaolinite, montmorrilonite, quartz etc.)
  b) sesquioxides (Fe, Al - (hydr)oxides) e.g. allophane, gibsite, goethite, hematite, ferrihydride etc.)

- organic matter
  a) low molecular weight compounds, e.g. fats, alkanes, wax esters etc.
  b) fulvic acid

c) humic acid

d) humin

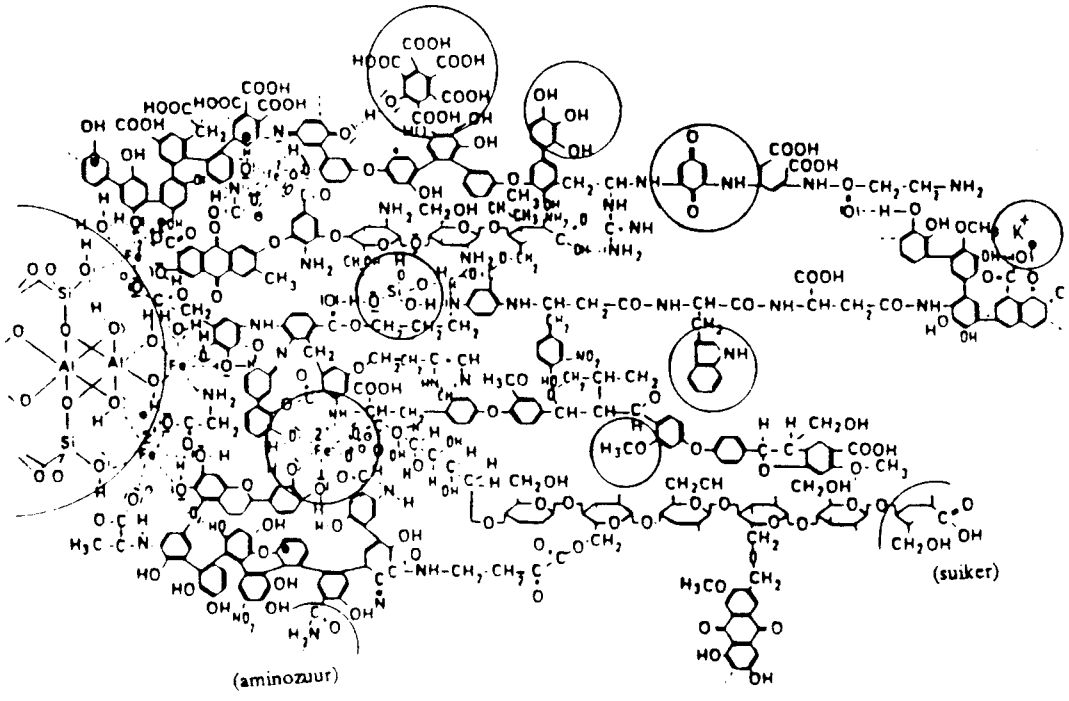

Fig. 1 Proposed model for the structure of soil organic matter [62]

- organic matter - clay complexes.

For some examples of the ranges in composition of the soil organic matter and organic matter clay complexes in various soils see Appendix I.

The structure of the paper was kept as close as possible to the one used in most articles on soil organic matter analysis. The structure is as follows:

1) Extraction techniques
2) Purification techniques
3) Fractionation techniques
4) Characterization techniques

The extraction part deals with methods used to get the soil organic matter separated from the soil mineral matter. The purification part is necessary because of the incomplete separation of the organic matter from the mineral matter, also extraction reagents contamination of the organic matter is taken care of. This means that purification is necessary to lower the ash content. Soil organic matter is too complex to be dealt with immediately so a fractionation step follows to get fractions that are more homogeneous in composition. After this the fractions can then be characterized by all kinds of methods described in the characterization part.

2. Extraction methods

Extraction of humic materials from soils by basic solvents is based on the increased dispersion of the humic acid mixed aggregates or micelles. At increased pH more humic micelles will be dispersed into solution, because more acid groups will be ionized. The resulting increased charge on the membrane surfaces will lead to disruption by repulsion of the larger structures and formation of smaller structures (e.g. micelles). This disruption consists of breaking up of the weak interactions (e.g. hydrogen bonding, π bonding, and hydrophobic interactions). In addition to weak interactions, stronger ionic interactions, such as those between metal ions and charged groups in the humic materials must also be disrupted [22].

2.1. Sequential extraction of organic matter

A method used more and more is a sequential extraction method for the extraction of organic matter from soils (Duchaufour and Jacquin, 1963 [58]; Smith and Lorimer, 1964 [59]; Gascho and Stevenson, 1968 [60]; Goh, 1970 [61]; Felbeck, 1971 [51]; Schnitzer and Schuppli, 1989 [36]). The advantage of using a sequential extraction method is that the obtained fractions may be more homogeneous than the material extracted by one extraction step only.
Felbeck [51] used the following sequence:

a) benzene(C_6H_6) - methanol(CH_3OH)
b) 0.1 N HCl
c) 0.1 M $Na_4P_2O_7$
d) 6 N HCl at 90 °C
e) 5:1 chloroform($CHCl_3$) - methanol(CH_3OH)
f) 0.5 N NaOH

Cameron et al.[7] used the following sequence:

a) 0.1 M $Na_4P_2O_7$
b) 0.5 M NaOH(20 °C)
c) 0.5 M NaOH(60 °C)

Schnitzer and Schuppli [36] used the following procedure (Fig. 2):

a) n-Hexane(C_6H_{14}) ⇨ extracts: alkanes, fatty acids.
b) chloroform($CHCl_3$) ⇨ extracts: fatty acids, long chain alcohols, wax esters.
c) 0.1 M $Na_4P_2O_7$ under N_2 ⇨ extracts: organic material complexed to metals and clays.
d) 0.5 M NaOH under N_2 ⇨ extracts: free organic material.
e) distilled H_2O ⇨ extracts: free organic material.

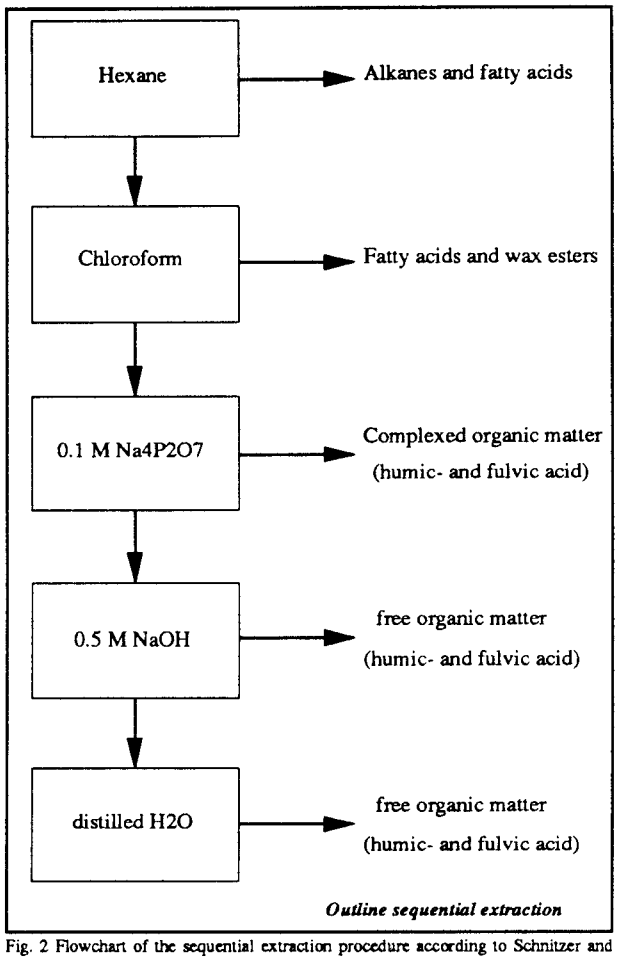

Fig. 2 Flowchart of the sequential extraction procedure according to Schnitzer and Schuppli [36].

The residual organic matter is assumed to be essentially humin.

When the methods proposed by Felbeck and Cameron are compared to the one proposed by Schnitzer and Schuppli it becomes clear that the former methods are not suitable for the extraction of soil organic matter. Step d (Felbeck) and step c (Cameron) could lead to major structural damage to the extracted soil organic matter. Schnitzer and Schuppli's method is more suitable for the extraction of soil organic matter because it extracts soil organic matter under much milder conditions.

The use of dilute alkali has been criticized because it could alter Organic Matter (OM) through hydrolysis and autooxidation [48]. On the other hand, a number of workers (Konova and Belchikova, 1961 [52]; Smith and Lorimer, 1964 [53]; Schnitzer and Skinner, 1968 [18]) have shown that there was little evidence to demonstrate that dilute alkali under an atmosphere of N_2 damaged or modified soil OM.

Step **a** and **b** of Schnitzer and Schuppli's method are based on dissolving the components from the total organic matter. For explanation of the principles behind step **d** and **e** (e is in fact a diluted alkaline solution because of residues of NaOH remaining after step **d**).

Extraction of OM with dilute solutions of neutral salts ($Na_4P_2O_7$) is based on the following reaction:

$$R\text{-}(COO)_4Ca_2 + Na_4P_2O_7 \rightarrow R\text{-}(COONa)_4 + Ca_2P_2O_7$$
$$\uparrow$$
$$\textit{soluble salt}$$

This extraction is most efficient at a high pH and high temperature.
From the reaction above follows that 0.1 M $Na_4P_2O_7$ is especially effective for the extraction of organomineral complexes without removing Fe and Al from soil forming parent materials. The use of $Na_4P_2O_7$ also eliminates the requirement to decalcify calcareous soils prior to extraction, as is the case with dilute NaOH.
Using ^{13}C NMR it could be shown that OM extracted with $Na_4P_2O_7$ was more aromatic than OM extracted with NaOH [57].
The fractions obtained (by means of the method proposed by Schnitzer and Schuppli [36]) were studied by means of the following techniques: organic C (carbon) (dry combustion) and total N (micro Kjeldahl method), E_4/E_6 (absorption) ratios, IR - spectrometry.

The following conclusions can be drawn from this method [36]:

- As each extract and residue resulting from this procedure can be characterized by means of chemical and spectroscopic methods, it is possible to determine quantitatively how much of the initial OM, and qualitatively which major components, have been extracted at each step of the procedure;
- The humic acids separated from the extracts and characterized by means of C content, E_4/E_6 ratio, and IR - spectrometry appear to have the same characteristics as typical soil humic acids (HA's). So the procedure does not seem to alter the HA's.
- The proposed extraction procedure is applicable to a wide range of soils. The procedure is relatively simple, N- and S - containing reagents which are often difficult to remove, as well as high temperature and harsh conditions are avoided. Where extraction yields are small fractions could be pooled, i.e. hexane + chloroform and H_2O + NaOH extracts.

According to Stevenson(1982) [48] the ideal extraction method should meet the following objectives:

a) it should lead to the isolation of **unaltered** materials;
b) the extracted humic substances should be free of **inorganic contaminants**;
c) the extraction should be complete, thereby ensuring that the fraction extracted is **representative** for the entire molecular weight range;
d) the methods should be **universally applicable** to all soils;
e) the extraction method should be relatively **simple**;
f) the method should **not be too time consuming** so that large numbers of soil samples can be handled.

The method proposed by Schnitzer and Schuppli [36] can not comply with all the objectives stated by Stevenson [48]. In fact there is not one extraction method that complies to all objectives. But nevertheless it should be possible to extract relatively unaltered representative materials. The method is also simple and universally applicable to all soils. However the organic substances extracted by this method are not free of inorganic contaminants and the method is rather time consuming.

2.2. Method for extraction without chemical alteration

Gascho and Stevenson [17] described a method which reduces the possibility of chemical alteration of soil organic matter during extraction. The essential features of the method are: (i) destruction of hydrated silicate minerals by pretreatment of the soil with 0.3 N HF, (ii) recovery of organic matter by extraction first with 0.02 M $Na_4P_2O_7$ and then with 0.03 N NaOH, and (iii) removal of inorganic contaminants by dialysis against 0.3 N HF.
In an attempt to facilitate the recovery of humic and fulvic acid free of mineral matter, the soil is first dialyzed against 0.3 N HF to destroy hydrated silicate minerals, after which the residue was dialyzed successively against 0.02 M $Na_4P_2O_7$ and 0.03 N NaOH to dissolve organic matter. By using dilute solutions of these reagents, very little of the extracted organic matter was broken down into low molecular weight constituents capable of passing through Visking dialysis tubing. Alternate dialysis of the extracted organic matter against 0.3 N HF and 0.02 M $Na_4P_2O_7$ gave humic acids with ash contents less than 2 %. Nearly all of the extracted material could be precipitated after acidification. This is so because dilute solutions of HF, $Na_4P_2O_7$, and NaOH do not disrupt intermolecular forces binding "fulvic acids" to "humic acids", which presumably occur through an ester type linkage. So the distinction between humic and fulvic acids appears to be rather artificial.

2.3. Method for the extraction of humin

Rice and MacCarthy [49] developed a method for the extraction of humin from streamsediment samples. This method consists of a series of steps that partition the organic substances in a sample (sediment, soil, etc.) between an aqueous phase and methyl isobutyl ketone (MIBK) according to their varying solubilities as a function of pH. The organic carbon of humin constitutes the largest fraction of the organic solid phase, representing as much as 80 % of the total organic carbon in the soil. From this follows that the extraction of soil organic matter without the extraction of humin does not give a representative picture of the organic matter in the soil. The problem with the extraction of humin from soil is that humin is insoluble in an aqueous system at any pH.
The humin isolated by the method of Rice and MacCarthy is obtained as the result of an active isolation step and is not simply the residue that remains after extracting humic acid and fulvic acid.

The method consists of the following steps:

a) A dried sample (1g of sediment, soil etc.) is added to 100 ml of 0.5 M NaOH solution and stirred for 24 h.
b) The entire mixture from step 1 is transferred to a separatory funnel along with 75 ml of MIBK and acidified to pH 1 with concentrated HCl.
c) The mixture is shaken vigorously and the organic matter is allowed to **partition** between the organic and aqueous phases. Humin and humic acid enter the MIBK phase as a **suspension**, leaving most of the fulvic acid in the aqueous phase. The aqueous phase containing most of the fulvic acid is discharged.
d) A fresh 100 ml aliquot of 0.5 M NaOH is added to the separatory funnel, the contents are shaken vigorously, and the organic matter is allowed to partition between the two phases. The humic acid is extracted from the MIBK phase into the aqueous alkaline phase. This separation leaves behind, **suspended** in the MIBK phase, a material that conforms to the definition of humin.

A review of the steps can be seen in the flowchart below:

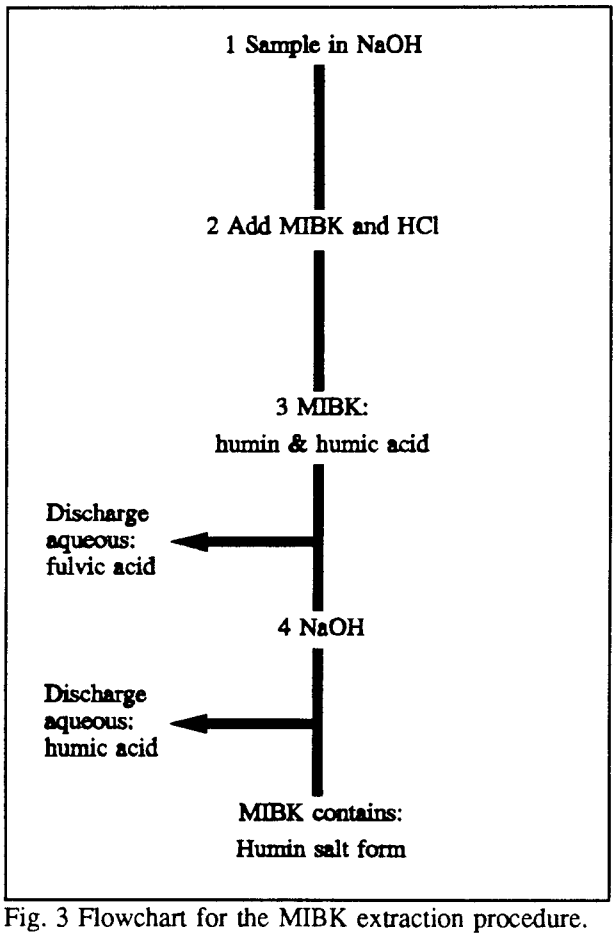

Fig. 3 Flowchart for the MIBK extraction procedure.

2.4. General review of other extraction methods available [21]

Schnitzer and Khan [21] gave a review of extraction methods available.

- for many years the NaOH extraction method under N_2 (usually in a soil:extractant ratio of 1:10) has been applied in soil organic matter extraction because it is a very efficient extractant. From the literature it followed that the NaOH solution is used in concentrations of 0.1 N or 0.5 N. No evidence exists to show that dilute alkali damages or modifies the chemical structure of humic materials. The method is widely used so it is easier to compare.

- $Na_4P_2O_7$ 0.1 N at pH 7 is also used as an extractant (see 2.1). The main problem with this type of extraction is that the pyrophosphate is difficult to remove from humic materials and might interfere during the characterization of the extractant.

- Various organic solvents (acetyl acetone, acetone-water-HCl system, anhydrous formic acid (HCOOH) + 10 % acetyl acetone, ethanol), dipolar aprotic solvents(pyridine, ethylenediamine) have been used in soil organic matter extraction. The danger in the application of these solvents is the irreversible adding of C and N to the humic materials.

2.5. Conclusions

The preference for a certain extraction method depends on the amount of detailed information required in soil organic matter research. The method proposed by Schnitzer and Schuppli [36] provides a representative and detailed picture of the soil organic matter without becoming too tedious and time consuming. Because of these reasons this method is recommended for soil organic matter extractions.

3. Purification

Purification is necessary to make the functional groups accessible to the reagents used in subsequent characterization steps.

Schnitzer and Khan gave a review of the possible purification techniques [21].

- Humic acids can be purified from inorganic constituents by shaking at room temperature with dilute solutions of HCl-HF (0.5 ml conc. HCl+0.5 ml of 48 % HF+99 ml of H_2O). After shaking for 24 to 48 hours, the acid mixture is removed by filtration and the residue is washed with distilled water until free of Cl^- and then dried.
- Dialysis of humic acid: salts and low molecular weight organic compounds are readily removed, the method cannot separate complexed or strongly adsorbed metals or metal hydroxides from humic materials.
- Purification can also be achieved by precipitation of humic acids at pH 2 and adsorption of fulvic acids on a small polyvinylpyrrolidone (PVP) column; this allows the purification of fulvic acids from pyrophosphate ions, metals, non phenolic organic substances. The adsorbed fulvic materials is washed with 0.01 N H_2SO_4 and eluted with 0.5 M NaOH. The purified solution of fulvic acids is combined with the redissolved humic acids and is then treated with Amberlite M^+ ion exchange resin.
- Schnitzer and Desjardins [5] used electrodialyzation of humic materials until the current fell to below 25 mA at a potential of 500 Volts.
- One can also use the method of repeated precipitation and dissolution of humic acids following the method of Cameron et al. [7]. Humic acids were recovered by precipitation with H_2SO_4 at pH 1 and purified by redissolving at pH 7 by the addition of NaOH, centrifuging to remove insoluble materials, reprecipitating at pH 1 and removal of the supernatant liquid. After repeating this process several times the humic acid precipitates were thoroughly dialyzed before freeze drying.
- Fulvic acids can be purified by passage over Amberlite IR-120 or Dowex-50 exchange resins in H-forms.
- Molecular exclusion gel chromatography using Enzacryl gel results in the removal of low molecular weight constituents (such as aliphatic, aromatic and phenolic acids) from high molecular weight humic acids and fulvic acids. Fulvic acids can be further purified by adsorption onto XAD-8 (a non-ionic, macroreticular acrylic resin) followed by elution over a H-resin.

4. Fractionation

There are several methods for fractionation of soil organic matter. In chapter 2 some sequential extraction procedures for soils have been described, while in this chapter some other fractionation techniques based on differences in molecular size, sorption properties and electrical properties are given.

4.1. Gel permeation chromatography

Gel Permeation Chromatography (GPC) is a technique used to determine molecular weight distributions of substances that do not interact with the gel permeation resin. When there are interactions between the compounds and the resin it is still possible to use this technique in the chemical fractionation of the compounds. In GPC a porous resin is used. The pores cover a fixed range in molecular sizes (weights). Small molecules will penetrate the resin deeper than larger molecules. When the column filled with resin is eluted the excluded and thus largest molecules will come out first. The smallest molecules will be eluted last. In this way it is possible to determine the molecular weight distribution of a sample that does not interact chemically with the GPC resin.

Molecular weight distributions of humic materials determined by gel permeation chromatography are, at best, of doubtful value. This is so because gel permeation chromatography can only predict molecular weight distributions accurately when humic acid molecules are uniform in shape and chemical structure [23]. In figure 4 the effect of molecular weight and chemical structure on the elution volume can be seen. Janson(1967) [24] has shown in his review of the literature on adsorption on Sephadex that there are two types of departure from ideal molecular sieving behaviour on Sephadex gels. Some solutes may be retarded in their movement through the gel by adsorption or attractive electrostatic interactions, whereas others will be excluded from the gel by repulsive forces and will move through the column more rapidly than normal.

Humic acids are principally aromatic in nature, containing phenol, quinone, acid, amine, alcohol groups and ether linkages. It has been shown that phenols, quinones, aromatic acids and other compounds with large number of π electrons interact strongly with Sephadex dextran gels. Brook and Munday(1970) [25] have shown that the hydroxyl, amino, or carboxyl groups of monosubstituted derivatives of phenols, anilines, and benzoic acids interact with ether linkage and the hydroxyl groups on the dextran chains. Gel-solute interac-

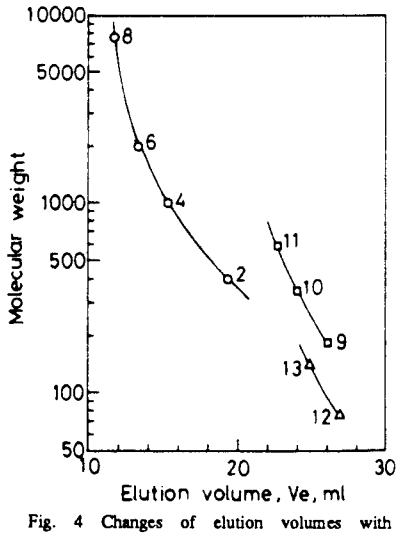

Fig. 4 Changes of elution volumes with molecular weight for Sephadex G-25 (●=polyethyleneglycols, □=sugars, △=glycines). This figure shows the relationship between molecular weight and elution volume and between chemical structure and elution volume [27].

tions fall into two main categories, the first caused by coulombic forces and the second to Van der Waals forces. Coulombic interactions, caused by charged sites on the gel and solute, are particularly strong when distilled water is used as eluant. The effect can usually be overcome by adding electrolyte to the elution system, or choosing the appropriate pH in the case of ionized acids or charged bases of the elution system, causing suppression of charges. This doesn't in all cases lead to satisfactory results. Especially in the cases of certain aromatic and phenolic compounds the addition of electrolyte has been found to increase adsorption(Gelotte, 1960; Demetriou et al.,1968). This effect can be explained with the fact that molecules containing hydrophobic portions are attracted to the gel phase where there is no salt and they are not completely surrounded by water. Hydroxyl and methoxyl substituents on an aromatic ring lead to increased adsorption on Sephadex(Demetriou et al., 1968). Determann and Walter(1968) showed that the gel-phenol affinity could be related to the ether bonds in the cross linking groups of the Sephadex matrix. Thus, as the degree of gel cross-linking increased so did affinity of phenol for the gel. Adsorption forces which are often caused by hydrophobic interactions, always lead to retention of the solute and are not so readily overcome.

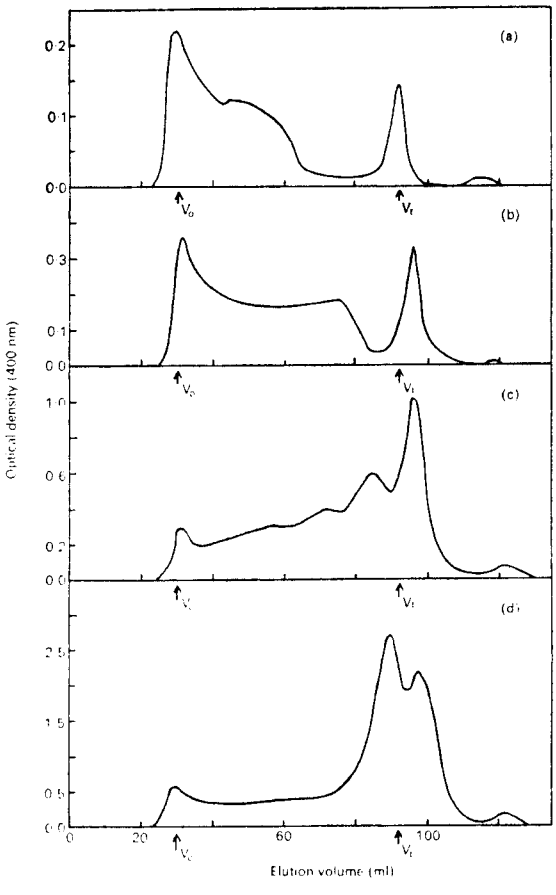

Fig. 5. Fractionation of humic acid on Sephadex G-100 at various sample concentrations using distilled water as eluant. Sample concentrations are: (a) 2 mg, (b) 4 mg, (c) 10 mg, (d) 20 mg per 2 ml [37], Vo = outside volume (this is the volume of the mobile phase outside the gel beads), Vt = outside volume (Vo) + inside volume (Vi) (volume of the stationary phase in the gel beads) + matrix volume (Vm) (volume of the bead material).

The type and degree of adsorption appears to depend largely upon the origin of the humic acid, the grade of Sephadex used, and the composition of the eluant [37]. Separation based solely on molecular weight differences implies that: a) the elution volume must be independent of the sample concentration and flow rate and b) the whole of the applied sample (i.e. the final peak) must be eluted within the total column volume. Figure 5 is an example of fractionation **not** solely based on molecular differences. These patterns can be explained the following way [37]. As the sample concentration decreases a greater percentage of the sample moves into the excluded and near excluded regions. Both the humic acid and the gel carry negative charges and, as the sample concentration decreases, the total ionic strength is diminished and the suppression of charge is decreased. The

charge double layers on the solute and the gel extend further into the solution, resulting in effectively larger solute molecules and smaller pore sizes. Charge repulsion effects therefore occur at greater distance leading to increased exclusion with decreasing sample concentration.

A good example of the way in which adsorption is strongly correlated to the degree of cross linking within the gel is figure 6. Sephadex G-50 has a much lower exclusion limit (molecules larger than the exclusion limit will be excluded from the gel) than G-100 and should exclude a larger proportion of the sample. As can be seen in figure 6b only a small fraction is excluded, the major part being retained well to the rear of the column.

Nevertheless one can use gel permeation chromatography to increase the chemical homogeneity of humic acid fractions. In order to obtain chemical fractionation the functional groups of various humic acid fractions must interact with the functional groups of the gel. The amount of interaction between a humic acid molecule and the gel will be dependent on the functional group composition of the molecule; therefore different types of molecules will move through the gel column at different speeds. The fractionation obtained in this way by gel permeation chromatography is probably due to the interaction of at least three different processes; molecular sieving, Van der Waals adsorption, and ion exchange.

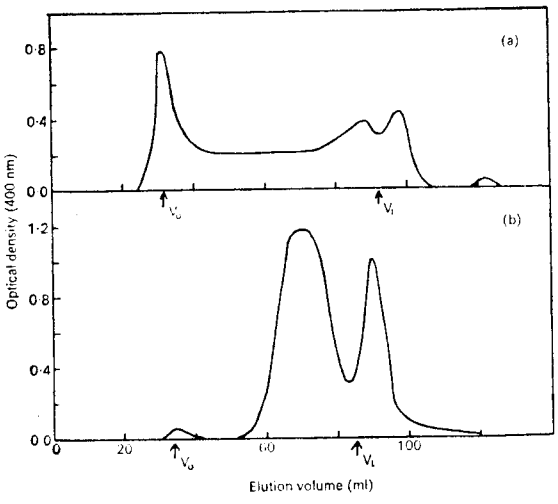

Fig. 6. Fractionation of (a) humic acid (NaOH extract, high molecular weight) on Sephadex G-100 and (b) humic acid ($Na_4P_2O_7$, low molecular weight) on Sephadex G-50 using distilled water as eluant [37].

4.2. Gel permeation chromatography without solute - gel interactions

The evaluation of the molecular weight distribution by means of gel permeation chromatography is according to Swift et al.[30] under certain circumstances, possible.

This is done the following way:

First the extracted humic acid is converted to the ammonium salt. This is done by exact neutralization to pH 7 with 0.5 N NH_4OH. The converted humic acid is then dissolved in 150 ml of buffer (0.05 M KCl, 0.02 M $NaHCO_3$, 0.001 M EDTA; pH=8.5). This solution is then eluted through a large, preparative, 12 % agar gel column (length=48 cm, diameter=14 cm). The colored eluant is collected as 200 ml fractions, a total of 50 fractions being required.

Agar is chosen as the gel permeation column packing because humic acid does not adsorb into this

material in buffered salt systems. The problems associated with the use of Sephadex gels under similar conditions are therefore avoided.

According to Swift and Posner,1971 [37] it is possible to obtain reliable molecular weight distribution results when Sephadex columns are eluted with buffered salt systems at an alkaline pH (borate or tris (2 amino-2(hydroxymethyl)-propane-1,3-diol)). The elution patterns so obtained will respond to changes in molecular weight in two ways (Fig. 7 a and b). The size of the excluded peak will vary with the percentage of high molecular weight material in the sample while the position of the maximum of the retained peak will reflect the average molecular weight of the material which can penetrate the gel pores. The pattern also complies with the requirements set for fractionation based solely on molecular weight: changing the sample concentration does not fundamentally alter the elution pattern and application of a sample of higher molecular weight led to increased exclusion. Also running the same sample on G-50 and G-100 showed greatly increased exclusion on G-50 as would be expected under ideal conditions. Differing the ionic strength produced negligible effects on the elution pattern indicating that ionic strength has little effect on Sephadex pore size. Thus, it is reasonable to suppose that gel-solute interactions have been minimized or completely eliminated.

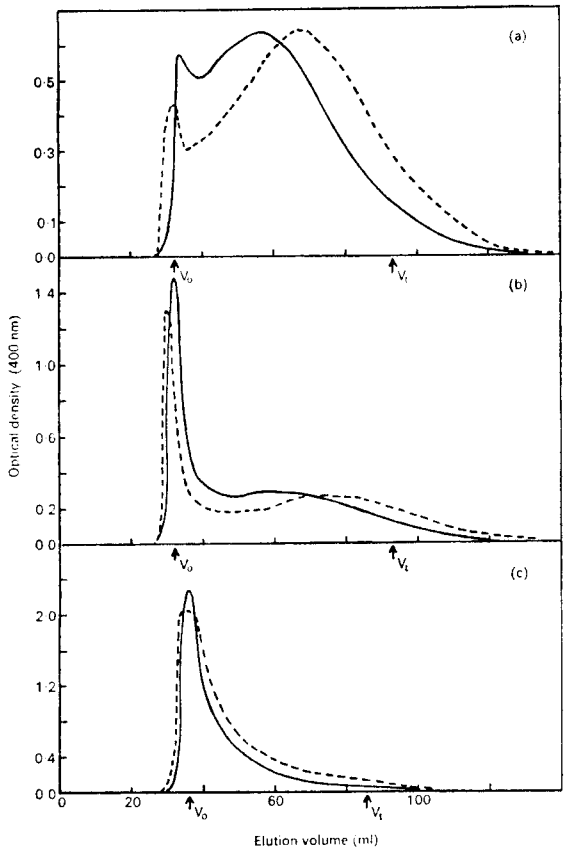

Fig. 7. Fractionation of (a) humic acid ($Na_4P_2O_7$ extract) on Sephadex G-100, (b) humic acid (NaOH extract) on Sephadex G-100 and (c) humic acid ($Na_4P_2O_7$ extract) on Sephadex G-50 [37].
——— Borate buffer eluant
---------- tris buffer eluant

In order to obtain a continuous fractionation on the basis of molecular weight within the column volume, the following conditions should be fulfilled [37]: the gel column should be packed and eluted with an alkaline buffer, preferably tris or one containing a similar amino cation. Bio-gel P and possibly Sephadex G gels should be used for the low and intermediate molecular eight humic acids and agarose based gels for the highest molecular weight fractions.

4.3. In situ fractionation theory

A means of fractionation often reported in literature [2], [5], [6], [50] makes use of the in situ fractionation assumption. The assumption being made, for soils with an O/Ao and B horizon, is the following: organic material from the O or Ao horizon is humic acid and material from the B horizon is fulvic acid, this fractionation is the result of prolonged leaching through these layers. It is of course doubtful to what extent this assumption holds, whether or not the organic matter fractionates in the soil so strictly into two distinct chemically different fractions. The method of course is not very laborious, because it only involves separation of the O/Ao and B horizons, and doesn't bear the risk of loosing, because of inefficient fractionation methods, or chemically altering organic matter.

4.4. Reverse phase liquid chromatography (RPLC)

On water soluble organic matter a completely different fractionation technique can be applied. The water soluble organic matter has gotten more and more attention lately, because dissolved organic matter (DOM) can act as sorbent for exogeneous organic chemicals in soils, possibly leading to enhanced leaching of these compounds [41], [42], [43]. The distribution of exogeneous organic chemicals in soils is considered as an equilibrium in a three compartment system: dissolved, sorbed to dissolved macromolecules, and sorbed to the bulk soil matrix. Reverse phase liquid chromatography was used to separate the complex DOM samples into distinct fractions based on relative polarity [15]. The instrument employed was a HPLC with an Econosphere 15 cm, 5μ C_{18} reverse phase column and UV detector set at 280 nm The mobile phase was comprised of an isocratic binary methanol:water mixture (50:50 v/v) which provided good resolution, at a flow rate of 1 cc/min. Relative polarity is defined in this study in terms of the affinity of the compound for the nonpolar stationary phase. The more polar a compound, the less the attraction for the column packing and hence, the shorter the retention time. Early peaks are, therefore, representative of more polar compounds.

4.5. Electrophoretic fractionation and isoelectric focusing

De Nobili et al. [1], [34] and Ceccanti et al. [13] used electrophoresis and electrofocusing to fractionate humic substances. Fractionation using electrophoresis is based on the difference in mobility of the various soil organic matter components in an electrically induced pH gradient. The differences in mobility are caused by differences in pK_a values of the functional groups in soil organic matter. Carboxylic - phenolic compounds focus with a slight band at pH values corresponding to their dissociation constants. One should always be cautious when interpreting electrophoretic/focusing patterns because of possible interactions of humic substances with carrier ampholytes.

These interactions cause increased heterogeneity with sample concentration. Preparative electrophoresis is done in acrylamide gel rods consisting of three different overlayed gels of decreasing acrylamide concentration (9 %, 7 % and 5 %) in phosphate buffer at 15 mA per rod. Runs were stopped when the front of the migrating band reached the bottom of the 9% gel segment. 2 cm long slices containing the front (fraction L), the centre (fraction M) and the tail (fraction H) of the migrating band of humic substances were cut from several replicate rods. Slices were extracted with 0.1 M $Na_4P_2O_7$. Extracted fractions were concentrated by adsorption on PVP columns. Electrofocusing of total extracts and of related fractions was carried out. Gels were scanned at 460 nm by means of a Varian 634 Vis Spectrophotometer equipped with a gel scanner.

Preparative electrofocussing cannot be used for the characterization of characteristic or common groups of bands because of:

1) doubts about the formation of complexes with the carrier ampholytes

2) humic substances are adsorbed at acid pH onto the dextran matrix of gels used as an anticonvective medium in preparative electrofocusing.

After extensive chemical and spectroscopical characterization of the fractions obtained the following conclusions were reached:

- comparison of fractions with the lowest electrophoretic mobility (fraction H) to those with the highest electrophoretic mobility (fraction L) IR-spectra show an increase in carboxyl group content and a corresponding decrease in ketonic and quinonic carbonyl content.
- similarities observed in the IR-spectra of corresponding fractions of humic substances of different origin were stronger than those found among fractions obtained from the same extract.
- fractions L, in accordance with their E_4/E_6 ratios, have the lowest molecular weight, and their electrofocusing profile practically coincides with that of nonexcluded humic molecules on a Sephadex G-25 column and with that of a fraction with molecular weight lower than 5000 daltons as obtained by ultrafiltration.
- soils developed under climatic conditions which favor fast mineralization do not contain appreciable amounts of substances with the electrofocusing behaviour of fraction H.
- humic substances that focus in different regions of the pH gradient differ as to their functional group content and to the amounts of bound saccharide or peptide residues in their structure.
- humic substances of widely different origin, but focusing in the same pH interval, do show evident structural similarities.

4.6. Fractionation of soil humic acids by adsorption chromatography

Yonebayashi and Hattori [10] recently used a new fractionation method. The technique is based on the fact that humic substances can be adsorbed on the nonionic macroporous resin Amberlite XAD-8 at pH 2 and could be separated into different fractions by pH gradient elution. Humic acids would be separated by the mechanism that as the pH of the eluent increases, the components having larger pK_a values would be progressively ionized and desorbed. The problem with soil humic acids is that they precipitate at pH 2. So that humic acids first had to be treated with Amberlite IR-120 resin in H^+ form to get the humic acids in H^+ form. The pH of these humic acids was about 3 and they did not precipitate when mixed with the universal buffer, an equimolar mixture of phosphoric, acetic, and boric acids, the pH of which was adjusted with NaOH. These humic acids were applied on to a column packed with Amberlite XAD-8 resin. A pH gradient solution was prepared by titrating 200 ml of 0.02 M universal buffer, contained in an air tight flask, with 0.1 M NaOH using a peristaltic pump. The pH of the effluent was measured with a pH electrode. Also a water - ethanol gradient was generated by mixing 200 ml water, contained in an air tight flask, with ethanol using a peristaltic pump. Elution was at a flow of 1.5 ml min^{-1}. The elution profile was determined by measuring the optical density at 400 nm after the effluent was alkalized above pH 12 by addition of 10 M NaOH.

The humic acid remained immobile until the pH rose to about 4. It was eluted between pH 4 and pH 11 in two peaks, each of which corresponded to one of the inflections in the pH-gradient curve. These embrace the pK_a values of the carboxylic and phenolic groups. The remaining humic acid in the column was eluted in one peak and a broad plateau using the water ethanol gradient. In order to improve the resolution, the adsorbed humic acids were first eluted in two steps with buffer solutions adjusted to pH 7 and to pH 11. Next the strongly adsorbed humic acids were eluted stepwise with water and then 50 % ethanol. The soil humic acid was thus separated into four fractions. These four fractions showed differences in elemental composition, functional group content, ^{1}H-NMR spectra and GPC chromatograms (the first eluted compound had a small excluded fraction and a large diffused fraction; therefore, the molecular weight distribution was narrow. The last eluted compound had a large excluded fraction and a small plateau in the diffused region; therefore, the molecular weight distribution was wide with a high average molecular weight). The method presented here with stepwise elution provides four fractions that are chemically different and have a different molecular weight distribution. It is very good possible to characterize different soil types by examining these four fractions. See also figure 8.

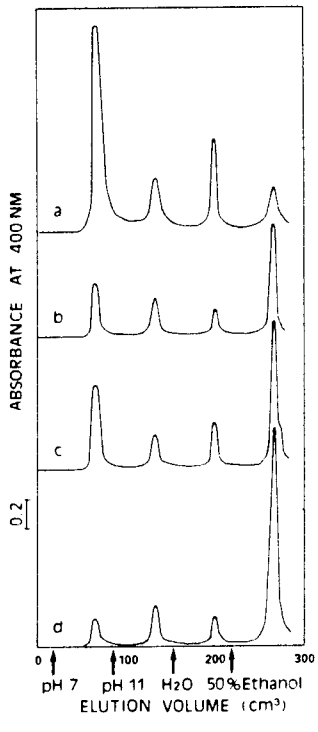

Fig. 8. Fractionation of soil humic acids by stepwise elution: a, Andisol; b, Entisol; c, Inceptisol; d, Histosol [10].

5. Characterization

After the fractionation of soil organic matter using techniques described in chapter 2 and 4, chemical and physical properties of the fractions can be determined for characterization. Several techniques can be used to determine the properties of the soil organic matter.
The following techniques are described: E_4/E_6 optical density ratio, pyrolysis GCMS, functional group (carbonyl, carboxyl, acidic hydroxyl) determinations and molecular weight determinations.

5.1. The E_4/E_6 ratio

In soil organic matter studies a parameter often measured [21], [1], [35] is the E_4/E_6 ratio, which is the ratio of optical densities or absorbances of dilute aqueous humic acid (HA) and fulvic acid (FA) solutions at 465 and 665 nm (E_4 = absorbance at λ = 465 nm, E_6 = absorbance at λ = 665 nm).
E_4/E_6 ratios of humic and fulvic acids are dependent on [35]:

i) the particle size (or particle or molecular weight)
ii) the pH
iii) the free radical concentration
iv) contents of O, C, COOH and total acidity in as far as these parameters are also functions of the particle size or particle or molecular weight.

E_4/E_6 ratios of humic and fulvic acids are **not** dependent on [35]:

i) the relative concentration of condensed aromatic rings
ii) the humic acid and fulvic acid concentrations, at least in the 100-500 ppm range.

Furthermore it has been suggested that light adsorption of aqueous HA and FA solutions in the visible region of the electromagnetic spectrum increases with [35]:
i) the ratio of carbon in aromatic nuclei to C in aliphatic side chains.
ii) the total C content.
iii) the molecular weight.

From experimental data [35] became clear that:

1. there is an inverse relationship between E_4/E_6 ratio and the molecular weight (M_w).

2. an increase in pH results in an increase in E_4/E_6 ratio, at very high pH a slight decrease in E_4/E_6 ratio. E_4/E_6 ratios for lower molecular weight FA fractions were especially sensitive to changes in pH, apparently because they contained **more COOH groups** per unit weight than did the higher molecular weight materials. Furthermore, the free radical content increases with pH.
3. there is an inverse relationship between reduced viscosities and E_4/E_6 ratios.

Point 2 and 4 above can be explained the following way [35]:

The pH increase causes the particles to become smaller and smaller, see Chapter 2. This manifests itself by increases in E_4/E_6 ratios and in free radical contents as the particles separate and disperse. At pH 7, FA particles in aqueous solution are already very small and further decreases in size with increases in pH no longer affect the magnitude of E_4/E_6 ratios. Free radical concentrations, on the other hand, continue to increase as the pH is raised because they become increasingly stabilized through the formation of semiquinone ions.

According to Doty and Steiner(1950) it is possible to obtain information about particle size by measuring the transmission at various wavelengths. They formulated the following relation for this purpose:

$$-\frac{d \log O.D.}{d \log \lambda} = 4-\beta$$

where

O.D. = optical density of suspension

λ = wavelength of transmitted light

β = a parameter which is a direct function of b/λ_0 with b = major dimension of particle, and λ_0 = wavelength of incident light.

Between the slope, of a plot of log O.D. and log λ, and E_4/E_6 ratios, the following relationship can be shown to exist:

$$-\frac{d \log O.D.}{d \log \lambda} = slope = \frac{\log E4 - \log E6}{\log 465 - \log 665} = -6.435 \log(E_4/E_6)$$

$$4 - \beta = -6.435 \log(E4/E6) \rightarrow \beta = 6.435 \log(E4/E6) + 4 \rightarrow$$

$$\beta \sim \frac{b}{\lambda_o} \rightarrow \beta = a \circ \frac{b}{\lambda_o} \; combinate \rightarrow$$

$$b = \frac{\lambda_o (6.345 \log(E4/E6) + 4)}{a}$$

$$a = proportionality\ factor$$

So the E_4/E_6 ratio is also a function of particle or molecular size which, in turn, is related to particle or molecular weight.

Attempts to use the above equation quantitatively to calculate particle or molecular dimensions of FA and FA fractions will not be successful because it is not possible to separate light adsorbed from light scattered and also because refractive indices of aqueous solutions of FA and FA fractions are too close to that of distilled water. But the equation can be used to provide one with a **comparative** idea of particle sizes in different FA preparations.

It can also be shown that there are correlations between E_4/E_6 and reduced viscosity (R=-0.95), %C (R=-0.73), %O (R=0.82), total acidity (R=0.62), COOH groups (R=0.66). A possible explanation for these correlations is:

A low E_4/E_6 ratio is associated with a relatively large molecular size or high molecular weight. This molecule has a large C - content, but is relatively low in O, COOH groups, and total acidity. A high E_4/E_6 ratio is indicative of the reversed.

It has frequently been suggested that E_4/E_6 ratios are indicative of the relative concentration of condensed aromatic rings. A low E_4/E_6 ratio is supposedly indicative of a relatively high concentration of condensed aromatic rings, a high ratio the reverse. But extensive structural studies [35] have shown that HA's and FA's behave like flexible, linear, synthetic polyelectrolytes implying that HA's and FA's must contain numerous linkages about which free rotation can occur. So that the occurrence of significant concentrations of aromatic structures exclusively or even largely composed of condensed rings is unlikely.

The **conclusions** that can be drawn from this are the following:

- the magnitude of the E_4/E_6 ratios is mainly governed by the particle sizes and weights of the studied materials.
- there are secondary relationships between the ratio and pH, O, C, COOH contents, and total acidity.
- there does not appear to be any direct relationship between the E_4/E_6 ratio and the concentration of condensed aromatic rings in HA's and FA's.
- the most favourable pH for measuring E_4/E_6 ratios for both HA's and FA's is between pH 7 and 8. This can be best achieved by dissolving between 2-4 mg of sample in 10 ml of 0.05 N $NaHCO_3$ solution. The resulting pH will be near 8.0. One should also use 0.05 N $NaHCO_3$ in the reference cell.

5.2. Pyrolysis-Gas chromatography(-Mass spectrometry) (PYGC or PYGCMS)

A number of workers have used pyrolysis-gas chromatography with or without mass spectrometry [1], [13], [33], [38]. Soil organic matter in its macromolecular state is difficult to examine. When these materials are subjected to rapid pyrolysis in an inert atmosphere they will

produce fragment molecules which may yield a characteristic chromatogram after GC separation. This chromatogram may serve as a "fingerprint" of the organic matter type in question. Also a variety of pyrolysis systems, conditions, and detection techniques have been used. Two pyrolysis systems are rather popular: in the past, the furnace type pyrolyser and nowadays the Curie point pyrolyser. The latter is the better one because it gives rapid and reproducible heating to the Curie point of the pyrolysis wire. Furthermore, wires of fixed composition give very reproducible pyrolysis temperatures and reproducible chromatograms. It also minimizes secondary reactions when both sample size and residence times of products in the hot zone are minimized. Generally, samples should be in the microgram range. Residence times in the hot zone in a Curie point pyrolyser with a carrier gas flow rate of 45 ml/min are 0.07 s, this in contrast to residence times of 2 s in furnace type pyrolysers. Special care should be taken in the purification of the organic matter extract because of the possibility of decomposition reactions being catalysed by iron oxides, alumina, etc. So differences in results using whole soils may reflect more than differences in organic matter. According to Simmonds et al. [54] proteins, peptides, and amino acids on pyrolysis yield nitriles (-C≡N), porphyrins give pyrroles, fats and waxes produce unbranched alkanes and alkenes, and carbohydrates yield aliphatic aldehydes, ketones and furan derivatives with $-CH_3$, -CHO, and $-CH_2OH$ substituents. So pyrograms containing much furans could reflect relatively undecomposed carbohydrates, whereas the pyrrole could be derived from the protein amino acids proline and hydroxyproline, structures containing porphyrin or the living biomass. Phenols may arise from lignin, its degradation products, or hydroxyphenyl groups in humic molecules [38]. Several pyrolysis ratios can be calculated [13]: furfural/pyrrole, furfural+5-methyl-furfural/pyrrole+cyclopentenone; both ratios can be considered as mineralization indexes: the higher the ratios the lower the degradation of vegetal material. Other ratios can also be considered: benzene/toluene, benzene/phenol+cresols, pyridine+o-xylene/phenol+cresols, pyrrole/phenol+cresols. These indexes increase when the organic matter is becoming more mature: the higher the ratios, the higher the humification. Often a similtude index is used to compare pyrograms. The similtude coefficient is a numerical parameter that permits a comparison between pyrograms without discriminating the peaks.

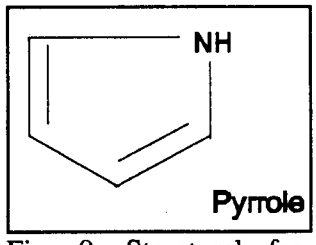

Fig. 9. Structural formula of Pyrrole.

$$Si_j = \sum^{n} \frac{\left(\frac{Ii^k}{Ij^k}\right)_{n=1}^{n=x}}{n} \qquad (Ii^k < Ij^k)$$

where I^k is the relative abundance of pyrolytic fragments of the i and j pyrograms considered, and n is the number of pyrolytic fragments considered.

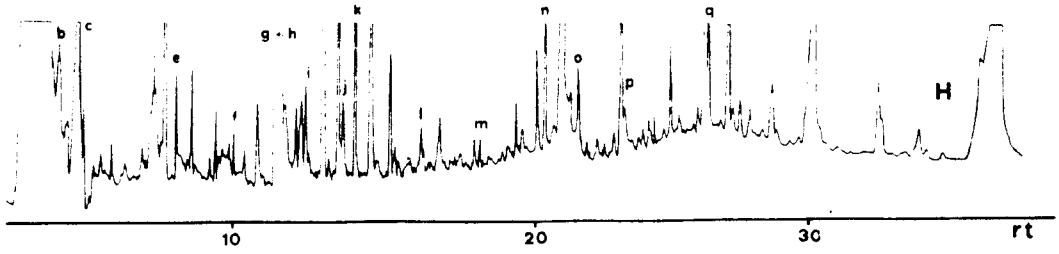

Fig. 10. Example of a pyrogram of humic substances [1].

A pyrogram is the gas-chromatographic representation of the pyrolysis products. When identification of the peaks is required each component (peak) has to be analyzed in a mass spectrometer; this produces a mass spectrogram. An example can be seen below.

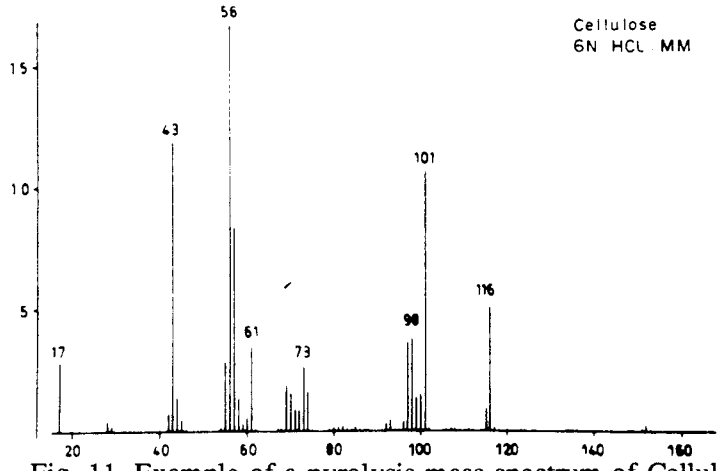

Fig. 11. Example of a pyrolysis mass spectrum of Cellulose [33].

With the help of a computer library program each component can be identified (when available in the library).

There are some problems concerning the usage of the similtude coefficient: a relatively low similtude level may be due to a few coincidences or to a greater number of coincidences, except for one or two main peaks. M. de Nobili et al. [1] used approximately the same pyrolysis ratios as B. Ceccanti et al. [13] used. They also come to the conclusion that PYGCMS plus the calculation of the appropriate ratios can make a useful contribution to the characterization and determination of the degree of humification of soil organic matter.

5.3. Determination of the Carbonyl group

The carbonyl group (aldehydic, ketonic, quinonic groups) of soil organic matter can be determined in several ways [2], [4], [6], [8], [11], [28]. Below a review is given of the various methods available.

The carbonyl groups can be determined by chemical and spectroscopic methods. The latter has the advantage that the sample stays more or less intact. A major setback in using spectroscopic methods is the requirement of relatively expensive equipment and experienced personnel necessary for the interpretation of the spectrograms. The techniques used are IR-spectrometry (produces

information on the occurrence and distribution of a number of characteristic groups) and more recently ^{1}H-NMR and ^{13}C-NMR for more quantitative measurements. The NMR techniques are especially useful when structural information of soil organic matter is required.

Simpler and less expensive techniques are the chemical determination techniques. Schnitzer and Skinner [2] have compared several derivative and titration methods. They formed four derivatives, that is, oxime, semicarbazone, phenylhydrazone and 2,4-dinitrophenylhydrazone. The formation of derivatives was checked by increase in nitrogen content(by the automated Dumas [45] method), IR, UV, and visible spectroscopy.

The formation of the **2,4-dinitrophenylhydrazone**:

$$R\text{-}C{=}O + H_2NNHC_6H_3(NO_2)_2 \rightarrow R\text{-}C{=}NNHC_6H_3(NO_2)_2 + H_2O$$

After the reaction has completed the solution is transferred to a dialysis bag and dialysed against distilled water for 100 hours. Following drying in a rotary evaporator, the air dry weight of the derivative is determined. From the blank run of 2,4-dinitrophenylhydrazine it became clear that approximately 50 % of the reagent had failed to dialyse and remained in the bag as reddish crystals. So there is a strong possibility that the derivative might contain appreciable amounts of unreacted reagent.

The amount of 2,4-dinitrophenylhydrazone formed is determined by the sensitive colorimetric method of Lappin and Clark [28]. This method is based on production of a intense wine red color on addition of base to an alcoholic solution of a 2,4-dinitrophenylhydrazone, due presumably to the formation of a resonating quinoidal ion. Under these conditions any excess reagent is converted into a light yellow substance, the adsorption of which is corrected for by using a blank determination.

The formation of the **phenylhydrazone**:

$$R\text{-}C{=}O + H_2NNHC_6H_5 \rightarrow R\text{-}C{=}NNHC_6H_5 + H_2O$$

After completion of the reaction the solution is transferred to a dialysis bag and dialysed against distilled water until free of chlorides. Following drying in a rotary evaporator, the air dry weight is determined.

The formation of the **semicarbazone**:

$$R\text{-}C{=}O + H_2NNHCONH_2 \rightarrow R\text{-}C{=}NNHCONH_2 + H_2O$$

After completion of the reaction the solution is dialysed against distilled water until free of chloride. Air dry weight is determined.

The formation of the **oxime**:

$$R\text{-}C{=}O + NH_2OH \rightarrow R\text{-}C{=}NOH + H_2O$$

After completion of the reaction the solution is dialysed against distilled water until free of chloride. Air dry weight is determined.

The amount of carbonyl groups in the organic matter is calculated from the increase in N content [2], [8] in the derivative compared to the original organic matter. The results of the four methods indicate that all methods by approximation result in the same C=O content. This indicates that quantitative estimates of C=O groups in soil organic matter can be made by methods such as have been used. One can also use this method without isolation of the derivative. But with a complex organic material such as soil organic matter, one is never quite sure whether under such conditions the reagent has (i) indeed reacted with the carbonyl groups ; (ii) reacted with other groups in the organic matter, or (iii) been adsorbed on the surface of insoluble organic matter and/or held in colloidal suspension by more soluble organic matter without reacting chemically in either case.

Reductive acetylation [2] was done in order to **determine the quinone group** content. To the organic matter acetic anhydride ($CH_3COOCOCH_3$), one drop of conc. H_2SO_4 and 2.5 g of zinc metal dust was added. The suspension was heated on the steambath for 24 hours, then filtered when hot. To clear the filtrate, 10 ml of distilled water was added and the solution heated on the steambath for 5 minutes to hydrolyze the acetic anhydride. Following this, the solvent was removed on a rotary evaporator. The dry residue was taken up in methanol and the solvent was removed. This procedure was repeated until the odor of acetic acid was no longer detectable. The product was thoroughly dried over P_2O_5 under vacuum and then prepared for IR analysis. In the IR spectrum, if the quinone groups have reacted, the typical C=O stretching vibrations were no longer detectable. New strong bands appeared , characteristic of the formation of phenolic acetates. This indicates reduction of quinone to phenolic OH groups followed by acetylation. So with this method it is possible, dependent on the type of spectrometric method applied, to determine either qualitatively or quantitatively the quinone groups in soil organic matter. Analysis results from Schnitzer and Skinner [2] indicate that the organic matter did not contain significant amounts of quinone groups.

According to Fritz et al. [11] it is possible to determine aldehydes and ketones by oximation in methanol-2-propanol, and subsequent backtitration with standard perchloric acid. Unlike most oximation procedures, the end point in this titration (determined either visually or potentiometrically) is very sharp.

When applying oximation with a hydroxyl-ammonium salt, several principles should be considered. The solution should be buffered at a neutral or slightly acidic pH during oximation. If this is not

done, the acid liberated may reduce the ratio of (NH_2OH) to $(NH_3OH)^+$ to a low value and result in incomplete or very slow oximation. A basic solution is avoided because of the instability of hydroxylamine in basic solution. Because oximation is subject to general acid catalysis, a high concentration of protonated species in the buffer will increase the rate of oximation. The reactions involved are as follows:

$$(NH_3OH)^+CL^- + B \rightarrow BH^+CL^- + NH_2OH \quad (1)$$

$$NH_2OH + R\text{-}C{=}O \rightleftharpoons R\text{-}C{=}NOH + H_2O \quad (2)$$

$$(Excess)NH_2OH + H^+ \rightarrow (NH_3OH)^+ \quad (3)$$

The difference between the titration of the excess base and a blank permits calculation of the amount of carbonyl present.

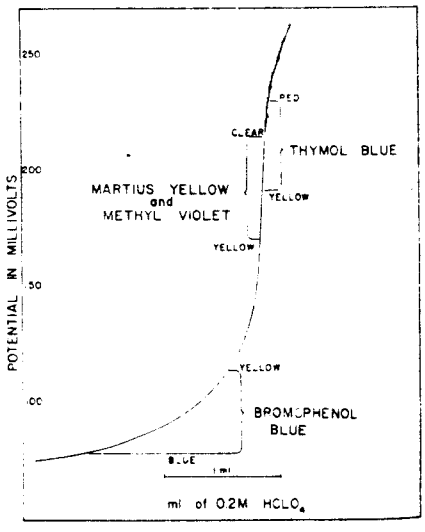

Fig. 12. Titration of blank showing indicator transition ranges [11].

The end point in the titration of a sample or blank may be detected either potentiometrically or by a visual indicator. Martius yellow mixed with a little methyl violet gives an excellent color change (yellow to colorless) that corresponds exactly with the potentiometric end point (figure 12).

A completely different method for estimating R-C=O is based on reduction to $R\text{-}CH_2OH$ with sodium borohydride ($NaBH_4$). Reduction is carried out in an alkaline solution, and H_2 liberated from the unused $NaBH_4$ is estimated manometrically. The method is reported to be highly specific for C=O groups [47], [46].

5.4. A Polarographic method for the determination of carbonyl groups

Schnitzer and Skinner [6] have developed a procedure to determine carbonyl groups quantitatively, this procedure consists of reacting the soil humic material with an excess 2,4-dinitrophenylhydrazine, followed by the polarographic determination of unreacted reagent. It has been shown that nitro groups in 2,4-dinitrophenylhydrazine could be reduced polarographically and that the height of the characteristic wave was proportional to the concentration of the reagent.
The polarogram obtained in this way is shown in figure 13. In figure 14 the relation between diffusion current and the concentration of 2,4-DNPH is shown.

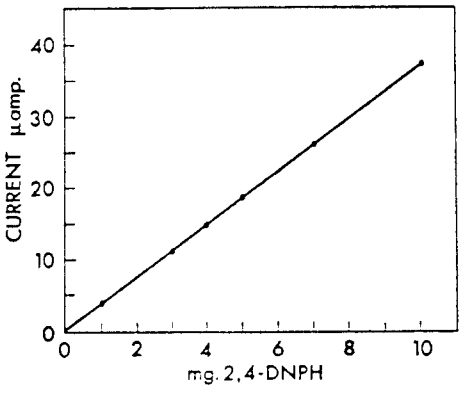

Fig. 14. Relation between diffusion current and concentration of 2,4-DNPH [8].

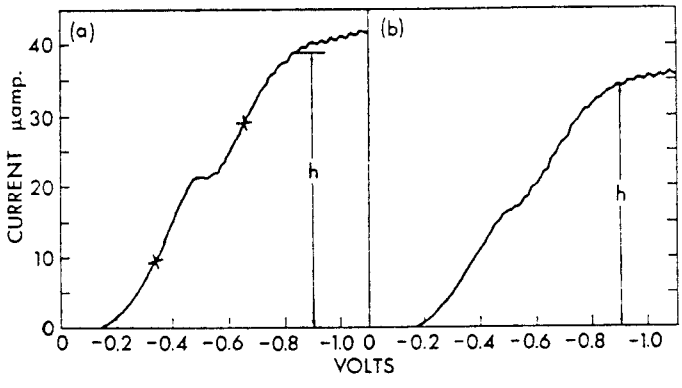

Fig. 13. Polarogram of (a) 1.0 milimolar 2,4-DNPH in ethanol, 0.04 N in H_2SO_4 [cross denotes the half-wave potential ($E_{1/2}$)], and (b) 2,4-DNPH in same solvent as (a) but after refluxing with soil sample and separation from the latter by filtration [8].

It was also noted that $E_{1/2}$ values were constant and independent of the concentration of 2,4-DNPH [8].
It was found that, because of the insolubility of soil humic compounds in ethanol-H_2SO_4, it was difficult to form derivatives by shaking humic compounds with excess 2,4-DNPH at room temperature. This procedure gave low results by comparison with analytical data obtained by oximation [11]. It was found most convenient to reflux the samples at the boiling point of the solvent system at approximately 75 °C. The most suitable period of time for refluxing appeared to be 30 minutes.

5.5. Determination of the COOH and acidic OH groups

The acidity of soil humic and fulvic acids is due to the presence of dissociable hydrogen in aromatic and aliphatic COOH and in phenolic and alcoholic OH groups. These groups can be determined by spectroscopic methods such as IR and NMR techniques. For these methods the same disadvantages as in 5.3 hold. The reactive hydrogens in these groups are responsible for the

exchange capacity of organic matter [3]. According to Schnitzer and Gupta [3] COOH groups can be determined by ion exchange with $Ca(OAc)_2$ and by a method based on a completely different principle, that is decarboxylation in quinoline with basic copper carbonate [29]. Total acidity [the sum of COOH + acidic(phenolic) OH groups] was determined by equilibration with $Ba(OH)_2$ and by discontinuous titrations. The content of phenolic OH groups was calculated by subtracting acidity due to carboxyls from total acidity. Special care must be taken to get the ash content to the lowest possible values in order to make the functional groups accessible to the reagents.

The $Ca(OAc)_2$ method is based on the following reactions:

$$(CH_3COO)_2Ca + 2\ R\text{-}COOH \rightarrow 2\ CH_3COOH + 2\ RCOO^-Ca^{2+}$$
$$CH_3COOH + NaOH \rightarrow CH_3COONa + H_2O$$

The $Ba(OH)_2$ method is based on the following reactions:

$$Ba(OH)_2 + \begin{matrix} 2\ R\text{-}COOH \\ 2\ R\text{-}OH \end{matrix} \rightarrow \begin{matrix} (R\text{-}COO)_2Ba \\ (R\text{-}O)_2Ba \end{matrix} + 2\ H_2O$$

$$Excess\ Ba(OH)_2 + 2\ HCl \rightarrow BaCl_2 + 2\ H_2O$$

Some problems with the application of the $Ca(OAc)_2$ method may be expected when strongly acidic OH (phenolic) groups are present, these groups will also participate in the ion exchange with $Ca(OAc)_2$ since these groups are dissociated [3], [46].

The decarboxylation method [29]: the carboxy group attached to an **aromatic nucleus** can be split off in the form of carbon dioxide by heating the acid with quinoline in the presence of a catalyst (basic cupric carbonate). The amount of CO_2 produced can be measured by recording the increase in pressure, or the increase in volume, or by weighing. The decarboxylation method is not a very precise method because some small errors, such as varying atmospheric pressure at the beginning and end of the determination, or different vapor pressure of the decarboxylated acid as compared to the acid, are inherent in this method [29]. Furthermore it is possible that CO_2 is released from α-hydroxy aliphatic acids during decarboxylation. Low and slow recoveries have to be expected when the decarboxylation method is used **also** for the determination of α-hydroxy aliphatic acids.

Because of the agreement between the decarboxylation method and the $Ca(OAc)_2$ method, Schnitzer and Gupta [3] concluded that the latter method is suitable for the determination of COOH groups in organic matter and is preferred to decarboxylation because of its simplicity. Bonn and Fish [14] on the other hand concluded that the acetate method cannot be expected to provide a "strong acidity index" that is constant over the range of reaction conditions that is typically employed. If, when reacted with acetate, the strongly acidic functional groups dissociate completely and the weakly acidic groups remain undissociated, then the measured carboxyl content will be an accurate index of strong acidity. Otherwise, the carboxyl content will vary with the HA/acetate ratio, reflecting the partial dissociation of the various acids. Like all other

titration methods, the acetate method distinguishes acidic functional groups by their relative acidity rather than their actual molecular structure. For this method to measure accurately the acidity due to carboxyl groups, the carboxyl acidity of the humic material must be quantitatively converted to acetate acidity. This will only occur if the following conditions are met when the humic acid/acetate mixture reaches equilibrium: (1) all humic carboxylic functional groups are completely dissociated, and (2) all other acidic functional groups in the humic material are completely undissociated. Because of the various positions of carboxylic functional groups in the humic macromolecule the acidity constant will not be a unique value but continuous distribution (titration data alone cannot uniquely determine a continuous pK_a distribution). Although the average carboxyl group will be more acidic than the average phenol group, the two distributions of pK_a will overlap. Some carboxylic and phenolic groups will therefore dissociate simultaneously. The acetate method is sensitive to any artefact (Ca-humate complexes, humic acid and humate) that alters the amount of base required in the titration of the filtrate. The formation of Ca^{2+}-humate complexes can significantly influence the amount of acetic acid that is present at equilibrium. When Ca^{2+}-humate complex is formed, the activity of the dissociated humate decreases, causing the humic acid equilibrium to shift towards the dissociated species. This shift causes an increase in the measured carboxyl content. When Ca^{2+}-humate complexation occurs, the carboxyl content value reflects the combination of two equilibria: acid dissociation and Ca^{2+} complexation. Perdue et al., 1980 [56] recommended the usage of an alkali acetate instead of $Ca(OAc)_2$. Alkali cations form very weak complexes with humate.
In conclusion the acetate method yields an index of strong acidity that is not unique and cannot be reproduced and compared unless **the exact experimental conditions (molarity of the acetate solution, mass and total acidity of the humic matter) are known**. It is also noted that the carboxyl content varies with the humic acid/acetate ratio. In view of the fact that the acetate method is sensitive to artefacts, titrations should be performed using an aliquot from the first 10 % of the filtrate. The carboxyl content is strictly an operationally defined value equal to the average degree of humic acid dissociation in a particular acetate solution.

Yet another method of estimating COOH groups is methylation and subsequent saponification of the resulting methyl esters [46]. Several methylation procedures have been used: CH_2N_2, methanol in dry HCl or H_2SO_4, and methyl iodide-silver oxide mixture. Several saponification procedures have been used: techniques based on titration of unused alkali with standard acid may give high results due to production of acidic groups during saponification.

From comparison with the discontinuous titration method it was concluded that the $Ba(OH)_2$ method appeared to be suitable for the determination of total acidity in soil organic matter [3]. The $Ba(OH)_2$ method is not specific for COOH and OH groups attached to aromatic rings; aliphatic COOH and OH groups will also react as long as the hydrogens dissociate under the experimental

conditions employed. Because of the above it is questionable whether one is justified to ascribe the difference between total acidity and COOH groups to the presence of phenolic OH groups in the organic matter. Yet there is some evidence [3] that these groups are in fact phenolic OH: (i) for all soil organic matter samples is the total OH (phenolic OH + alcoholic OH) as determined by acetylation [46] 1.5 to 2 times as high as OH determined by difference, (ii) IR-spectra showed broad adsorption probably due to phenoxy C-O vibrations of phenolic OH groups.

5.6. Nonaqueous titration of functional groups

Yonebayashi and Hattori [16] used a completely different method to determine functional groups in humic acid. Humic acids were titrated (under N_2) by CH_3ONa in $(CH_3)_2SO$ (DMSO), containing 0.05 mmol of benzoic acid and phenol as internal standards, using a platinum-calomel electrode system. The solution is titrated until the cell potential reaches a maximum. The equivalence point was determined from the differential titration curve.

DMSO (dimethylsulfoxide) was chosen as the solvent because H^+-humic acid is readily soluble in dry DMSO. A number of carboxylic and phenolic compounds whose pK_a values are known were titrated using this method. Figure 15 shows the relation between pK_a values and points of inflection. These were classified into three groups: the first and the second were the carboxyl groups and the last was the phenolic hydroxyl group whose pK_a values were <3.2, 3.2-7 and 7-10, respectively. So in a mixture of carboxylic acids and phenols the carboxylic and phenolic groups were titrated at the same time producing one inflection for each group.

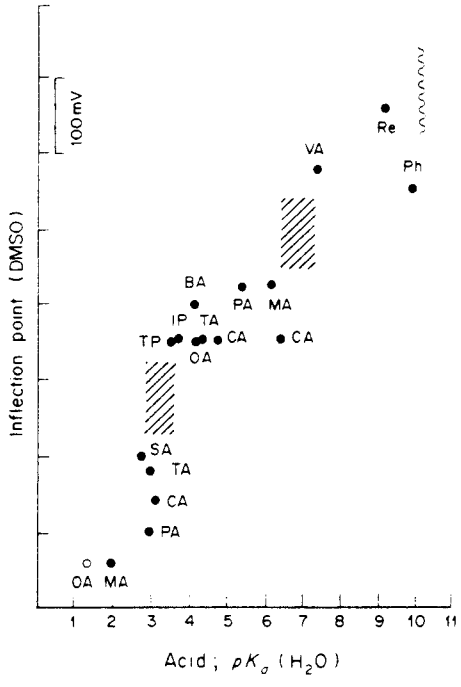

Fig. 15. Relationship between inflection potentials in nonaqueous titration and $pK_a(H_2O)$ values of various acids. OA-Oxalic acid; MA-maleic acid; PA-phtalic acid; CA-citric acid; TA-tartaric acid; SA-salicylic acid; TP-terephtalic acid; IP-isophtalic acid; BA-benzoic acid; VA-vanillic acid; Re-resorcinol; Ph-phenol [16].

When humic acid was titrated using this method without internal standards only one inflection appeared, but when the internal standards were added two inflection points appeared. This means that it is possible to titrate carboxyl groups and phenolic hydroxyl groups of Soil Organic Matter in the presence of benzoic acid and phenol as internal standards The effect of water in DMSO (up to 1 %) was not detected but water contents of more than 2 % resulted in the decrease of the second inflection and the increase in noise in the differential titration curve. So it is crucial that the best grade of DMSO is used after dehydration with molecular sieves. Different amounts of humic acids were titrated. The results showed that the titers for carboxyl and phenolic hydroxyl groups of the less humified humic acid were directly

proportional to the quantity. However results for the well humified material were not directly proportional to the quantity. This may be due to coagulation of humic acid by benzene which is present in the sodium methoxide. When the benzene content could be kept under 25 % than the titers are proportional to their quantity.

When carboxyl groups of low pK_a values are to be determined one should use phtalic acid as the internal standard. If humic acid is mixed with phthalic acid and titrated, the titer for the first inflection point must correspond to the carboxyl groups of low pK_a values of phthalic acid and humic acid. Results indicated that the titers for low pK_a carboxyl groups of humic acids were directly proportional to the quantity. Finally it should be mentioned that the nonaqueous titration methods which use internal standards have the following features:

1. these procedures were of microanalyses with high precision.
2. due to distinct inflections in titration curves, the equivalent points can be clearly determined.
3. the pK_a values of fractionally determined functional groups can be measured.

5.7. Molecular weight and size determination

Schnitzer and Khan [21] divided the methods available for molecular weight determination into three classes:

1) those measuring number average (M_n) molecular weights
   a. osmotic pressure
   b. cryoscopic
   c. diffusion
   d. isothermal distillation

$$M_n = \frac{\Sigma N_i M_i}{\Sigma N_i}$$

N_i = ***number of molecules***
M_i = ***molecular weight of the molecule***

2) those determining weight average (M_w) molecular weights
   a. viscosity
   b. gel filtration
   c. ultracentrifuge

$$M_w = \frac{\Sigma N_i M_i^2}{\Sigma N_i M_i}$$

3) those measuring z-average (M_z) molecular weights
   a. sedimentation
   b. ultracentrifuge

$$M_z = \frac{\Sigma N_i M_i^3}{\Sigma N_i M_i^2}$$

In a homogeneous material $M_n=M_w=M_z$, while in a heterogeneous material $M_n<M_w<M_z$ [5].

Factors influencing molecular weight determinations:
- differences in origin
- differences in extractants
- differences in the degree of purification
- pH
- ionic strength

The reasons for measuring molecular weight (see also 6. Applications):
a) to establish molecular formulae in conjunction with ultimate and functional group analysis.
b) to convert weight to molar concentrations, this is especially important in the study of organo-metallic interactions.

A number of methods have been reported in literature [5], [7], [19], [21], [30]. Cameron et al. [7] used a very elaborate and precise method to determine the molecular weight distribution of soil humic acid extracts. Whole humic acid extracts are usually too polydisperse for reliable molecular weight measurement. So in order to lower the polydispersity significantly the extract was fractionated by extensive use of gel permeation chromatography. The humic acid was converted to the ammonium salt, was applied onto an agar gel column and was eluted using a carbonate/bicarbonate buffer (0.02 M $NaHCO_3$+0.05 M KCl+0.001 M EDTA; pH 8.5, ionic strength=0.08). The second stage of fractionation was done on a Sepharose 6B gel column, eluted with tris buffer. Only the central portion of the peak was recovered and reapplied to the column, the tail portion of the peaks were discarded. This process was repeated 3 to 4 times until the reduction in polydispersity was considered satisfactory. The whole of the fractionation procedures can be seen in figure 16. The sedimentation coefficient of the humic materials were determined after equilibration with the buffer using a Spinco Model E ultracentrifuge fitted with

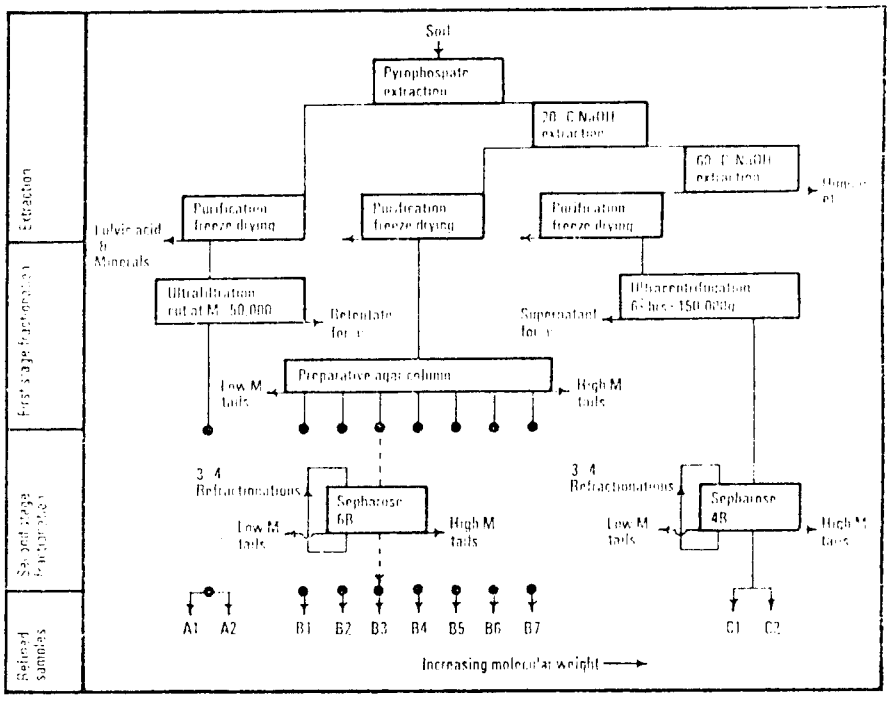

Fig. 16. Flow chart illustrating the complexity of the preparation of refined humic acid fractions [7].

Schlieren optics for measurement of concentration gradients. The synthetic boundary technique was employed using a 4°, 12 mm synthetic boundary cell containing 0.4 ml of humic acid solution overlain by 0.2 ml of tris buffer B (equilibrium dialyzate). Because of the low concentrations of humic acid used, the ionic strength should be high enough for charge effects to be suppressed. Diffusion coefficients were determined by a modified free boundary method. A sharp boundary was formed by carefully layering clear buffer on to dilute humic acid solution (approx. 0.1g/l) in vertical narrow bore (3mm in diam.) glass tubes using a small syringe. The formation of a sharp boundary was facilitated by having 30 % D_2O in the lower layer. The concentration of the diffusing humic acid was monitored by scanning the transmission of light throughout the length of the tube at various intervals of time using a Densicord scanning densitometer fitted with a blue light filter. The calculation of the diffusion coefficients was shown to yield approximately a weight median value for a polydisperse system. The partial specific volumes of the tris-humate were determined using a normal pycnometer method.

From all these data one can calculate the molecular weight using the Svedberg equation:

$$M = \frac{RTs}{(1-\bar{\nu}\rho)D}$$

where R=gas constant, T=temperature, s=sedimentation coefficient, ν=partial specific volume of the humic acid, and ρ=density of the solution

Thurman et al. [19] used small angle X-ray scattering to measure the range of particle sizes present in soil humic acid solutions. Aqueous solutions of sodium-humate (pH 12.5 and 7.0) were placed in a quartz glass capillary tubes and irradiated with Cu-K radiation on a Kratky small angle X-ray scattering goniometer using a 150 μm entrance slit and a 400 μm receiving slit. After numerous statistical correction the scattered intensity data can be analyzed by the method of Guinier [55]. Guinier has shown that for an ensemble of randomly oriented, identical scattering particles in which there is no long range order the scattered intensity may be approximated by the equation:

$$\ln I(h) = \frac{-h^2R^2}{3} + constant$$

where I(h)=scattered intensity, $h = 2\pi\sin 2\Theta/\lambda$ (2Θ=scattering angle, λ=the wavelength of the impinging X-ray), R=radius of gyration. The radius of gyration is defined as the root mean square distance of the electrons in the particle from the centre of charge. From the above equation it is seen that the radius of gyration may be calculated from the slope of a plot of ln I(h)/versus h^2 (Guinier plot). In a monodisperse system the Guinier plot is a straight line, in a polydisperse system the Guinier plot is concave. If there are only a few widely different sizes of particles in the

system, it may be possible to discern discrete straight-line segments of the curve, and these may be used to calculate radii of gyration of the different particle sizes. In general, a Guinier plot of a polydisperse system will only provide information on the range of particle sizes present in the system, not the distribution of sizes. It is important to consider that humic matter in water is associated with various metal ions, clays, and amorphous oxides of iron and aluminum. The isolation and separation process changes these confirmations and, indeed, may change the molecular size.

Schnitzer and Desjardins [5] determined the number average molecular weight by freezing point depression in sulfolane (tetrahydrothiophene-1,1-dioxide). Sulfolane was chosen because: (a) its cryoscopic constant (66.2 degrees - kg. per mole); this permits accurate measurements of freezing point depression even at very low solute concentrations; (b) it appears to eliminate disturbing effects due to association and/or dissociation of solute molecules; (c) it permits effective drying of hygroscopic materials because of the large difference in the boiling points of sulfolane (278 °C/700 mm; 100 °C/0.01 mm) and water, the latter can be removed by vacuum distillation prior to freezing point determinations; (d) it freezes at approximately 28 °C. Apparent molecular weights are calculated from the expression:

$$Y = \frac{1000KW}{w\theta}$$

where Y=the apparent molecular weight; K=cryoscopic constant; W=total weight of solute in grams; Θ=the observed freezing point depression; w=weight of solvent in grams. To obtain molecular weights, apparent molecular weights (Y) were plotted vs. concentration of organic matter (X), the drawing the line best fitting the experimental points with slope (C) and intercept M (molecular weight). In the limit of zero concentration Y=M.

6. Applications

Molecular size(weight) and shape play a role in the interaction with metals or other organic substrates. This interaction depends on the ability of metals and organic substances to penetrate the proposed random coil matrix, a process which is to a considerable extent regulated by the cations neutralizing the charges and by the water content of the medium. On acidification the charge on ionized groups is neutralized and inter- and intramolecular hydrogen bonding causes shrinkage with exclusion of solvent from the matrix, leading to precipitation of the organic matter. Polyvalent cations have a similar effect, drawing the structure together by bridging two or more negatively charged groups [20]. Furthermore it appears that ionic carboxylic functional groups greatly increase the size of humic substances compared to hydroxyl functional groups without increasing molecular weight. Probably this is due to hydration of carboxyl functional groups and possible mutual repulsions and expansion of the molecule [19]. Molecular size of a molecule is determined for the most part by the molecular weight, but deviations from this relation are possible due to the processes (pH effect, cation content, water content, hydration of carboxylic groups) mentioned above.

One of the applications of a soil organic matter characterization method is to see whether there is a relationship between certain soil organic matter characteristics and the adsorption behaviour of pesticides in the soil. For this it is necessary to have more knowledge on the chemical characteristics of the pesticides and on the adsorption processes between pesticides and soil organic matter. Below a review [21] is given of these characteristics and processes.

The pesticides used in agriculture can be divided into three categories:

1) Nematicides ⇨ controlling soil nematodes
1) Herbicides ⇨ controlling weeds
2) Insecticides ⇨ controlling insects
3) Fungicides ⇨ controlling plant pathogens

The interaction of pesticides with soil organic matter is an important factor affecting the fate of pesticides in the soil environment. This interaction has influence on the: degradation, bioavailability, leachability and volatility of pesticides.
The organic matter - pesticide interaction can express itself in two ways:

1) Adsorption of pesticides by organic matter. Adsorption will control the quantity of a pesticide in soil solution, and thus determine its persistence, mobility, leaching and bioavailability. The extent of adsorption depends on the nature of the chemical itself, on the kind and amount of soil organic matter and the environment provided in the soil.

2) Nonbiological degradation of pesticides by organic matter. Biological degradation of pesticides(-residues) by micro organisms (MO) is possible in several ways: degradation of "free" pesticides by MO as a primary substrate, and degradation of "bound" pesticides by MO as a secundary substrate, using the organic matter as the primary substrate.

One of the main characteristics of common pesticides is that most of them are low molecular weight compounds with low water solubility.
The following characteristics of pesticides influence the adsorption-desorption processes:

1) Chemical character
2) Shape
3) Configuration
4) Acidity (pK_a) (if it is of acidic character)
5) Alkalinity (pK_b) (if it is of alkaline character)
6) Water solubility
7) Charge distribution on the cation (if it is of cationic character)
8) Polarity of the molecule
9) Molecular size
10) Polarizability

Four structural factors determine the chemical character of a pesticide:

1) Nature of functional groups: -COOH, -C=O, -OH, and NH_2.
2) Nature of substituting groups that may alter the behaviour of functional groups.
3) Position of substituting groups with respect to the functional groups which may enhance or hinder intermolecular bonding.
4) Presence and magnitude of unsaturation in the molecule, which affects the lyophilic-lyophobic balance.

The charge characteristics of a pesticide are probably the most important properties governing the adsorption. This charge may be due to unequal distribution of electrons or dissociation. The pH of a system is important as it determines the ionization of the molecules. The pH is also crucial in explaining the differences in adsorption of acidic and alkaline pesticides. Neutral pesticides may be subjected to temporary polarization in the presence of an electric field. The availability of mobile electrons, such as π electrons in the benzene ring, influence the polarization of a neutral molecule.

Mechanisms of adsorption:

1) Van der Waals attractions ⇨ the only mechanism in the adsorption of nonionic, nonpolar molecules or portions of molecules.

2) Hydrophobic bonding ⇨ nonpolar pesticides or compounds with significant hydrophobic parts are likely to adsorb onto the hydrophobic regions of soil organic matter. Hydrophobic bonding is a form of Van der Waals attraction facilitated by gain of entropy by exclusion of the nonpolar pesticides from the water. Hydrophobic bonding is rather important for the adsorption of e.g. DDT and organochlorine insecticides onto the lipid fraction of soil organic matter and humus.

3) Hydrogen bonding ⇨ the presence of oxygen containing functional groups, as well as amino groups, on organic matter indicates that adsorption could occur by formation of a hydrogen bond with organic pesticides containing similar groups. For example, carbonyl oxygens on pesticide molecules may be bound to amino hydrogens or hydroxyl groups on the organic matter. Additional sites for hydrogen bonding by soil organic matter include -SH and -O- linkages.
It has been observed that hydrogen bonding may take place between C=O groups of the humic compounds and the secondary amino group of s-triazines.
Non-ionic pesticide adsorption at pH values below their pK_a values can be attributed to adsorption of the non-ionized form of the molecule on organic surfaces. Thus, hydrogen bonding may take place between the COOH group and C=O or NH group of the organic matter. Hydrogen bonding would be limited to acid conditions where COOH groups are unionized.

4) Charge transfer ⇨ the formation of charge transfer complexes is based on the electrostatic attractions induced by charge transfer between electron rich donors and electron deficient acceptors. The formation of charge transfer complexes has been postulated as a possible mechanism involved in the adsorption of s-triazines onto soil organic matter. Charge transfer complex formation may be detected by IR-spectrometry as a shift in the out-of-plane C-H vibration frequencies.

5) Ion exchange takes place for those pesticides that are either cations or become cations through protonation. Cationic pesticides are adsorbed via cation exchange functions through COOH and phenolic-OH groups. This adsorption is always accompanied by the release of a significant amount of hydrogen ions.
One can use IR-spectrometry to check the interaction between cationic pesticides and COOH groups. The effect on the IR-spectrogram is a decrease in the COOH absorption band and a subsequent increase in the COO^- absorption band. The cationic adsorption mechanism is also responsible for the adsorption on organic matter of less basic pesticides, such as s-triazines. The

pesticide may become cationic through protonation, either in the soil solution or during adsorption. Thus, a weakly basic pesticide may be protonated and adsorbed on organic matter according to the following equation:

$$P + H^+ \rightleftharpoons PH^+$$

where P=weakly basic organic pesticide.

When the solution pH is equal to the pK_a of the compound, 50 % of the basic pesticide molecules are protonated. In this case the pK_a is derived from the expression:

$$K_a = \frac{[H^+][P]}{[PH^+]}$$

Maximum adsorption of s-triazines by organic soil colloids occurs at pH levels near the pK_a of the respective compound. Thus the adsorption capacities of organic matter and humic substances for s-triazines were found to follow the order expected on the basis of pK_a values for the compounds (s-triazines). The pH of the soil solution influences the ionization of the acidic functional groups which affects the capability for cation exchange and it influences the adsorption of weakly basic pesticides. Reduction in solution pH results in an increase in the protonated species. For the subsequent adsorption of PH^+ it should compete with initially adsorbed cation M^+.

$$PH^+ + MR \rightleftharpoons M^+ + PHR$$

where R is the organic matter cation exchanger.
It has been shown that ion exchange could take place between a protonated secondary amino group on s-triazine and a carboxylate anion on the HA.

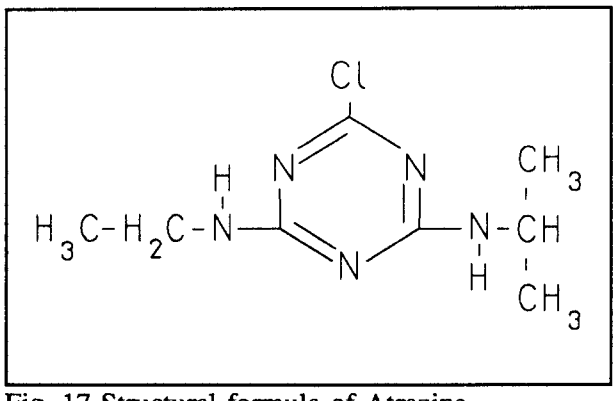

Fig. 17 Structural formula of Atrazine

6) Ligand exchange ⇨ adsorption by this mechanism involves replacement of one or more ligands by the adsorbent molecule. The necessary condition is that the adsorbent molecule be a stronger chelating agent than that of the replaced ligands. Also this type of binding is probably involved in binding s-triazines. In ligand exchange partially chelated transition metals may serve as possible sites for adsorption. The pesticide molecule may displace water of hydration acting as a ligand.

7. Final conclusions

The preference one has for a certain method, or pretreatment, often depends on the requirements set by subsequent stages in the characterization. So it is not possible to recommend just one extraction method, purification method, fractionation method and characterization method.

As far as the extraction techniques are concerned the sequential extraction technique as proposed by Schnitzer and Schuppli [36] seems a good choice. This method has the advantage that it provides the user with more homogeneous fractions than one would obtain using only NaOH. This means that it is possible to get a more detailed picture in the subsequent characterization of the fractions.

Purification techniques are as numerous as there are researchers and the preference for one method depends, especially here, on the requirements set by subsequent stages in the characterization.
Humic acids can be purified from inorganic constituents by shaking at room temperature with HCl-HF. The residue must be made free of Cl^- by washing with distilled water or extensive dialysis. The adsorbed cations can be removed by passage over Amberlite IR-120 in H^+ form. Fulvic acids can be purified by passage over Amberlite IR-120 in H^+ form or over PVP columns.

Fractionation is especially interesting when more homogeneous fractions are required. In order to achieve maximum fractionation gel permeation chromatography [23] is necessary for NaOH extracts, although it is also useful for the fractions obtained from the sequential extraction procedure. When one is dealing with dissolved organic matter (DOM) RPLC (reverse phase liquid chromatography) can be used to fractionate the DOM on basis of relative polarity.
A new technique for the fractionation of humic acids is adsorption chromatography [10]. This technique produces in a relatively simple way four chemically and structurally different fractions. The fractions also have a different molecular weight distribution.

Characterization of the Soil Organic Matter fractions can be quantitative or qualitative. For a **quantitative** characterization, the following functional group determination methods can be used.

Carbonyl group determinations through derivative formation (semicarbazone; oxime + possibly titration [11]; 2,4-dinitrophenylhydrazone; phenylhydrazone) and subsequent measurement of the increase in N-content. Another very convenient method is, the polarographic determination of carbonyl groups [8].
COOH group and OH group determination via nonaqueous titration [16]. **Total acidity** via reaction with $Ba(OH)_2$.

A **qualitative** characterization, as far as functional groups are concerned, can be achieved by IR spectrometry.

Molecular size (weight) distributions can be best determined by using the Gel Permeation Chromatography method "without" solute gel interactions (see Chapter 4.2).

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Appendix I Examples of organic matter compositions

This appendix gives some examples of the ranges in composition of the soil organic matter and organic matter clay complexes in various soils. Tables 1 and 2 show general composition ranges of some general compound groups. Tables 3, 4, 5, and 6 give a more detailed picture of the variations in composition within some general compound groups, in various soils.

Table 1 Waksman's Proximate Method of Analysis of Soil Organic Matter [64]

Fraction	Treatment	% of organic matter
Fats, waxes, oils	Ether extraction	0.5 - 4.7
Resins	Alcohol extraction	0.3 - 3.0
Hemicellulose	Hydrolysis (2% HCl)	5 - 12
Cellulose	Hydrolysis (80% H_2SO_4)	3 - 5
Protein plus "lignin-humus"	Analysis of final residues for C and N	
a. protein		30 - 35
b. "lignin-humus"		30 - 50

Table 2 Amounts of organic constituents as percentages of total OM in various soils [48]

Type of material	Extractant	Organic matter extracted (%)
Humic substances	NaOH	to 80 %
	$Na_4P_2O_7$	to 30 %
Amino acids, amino sugars	hot 6 N HCl	25 - 45 %
Sugars	hot 1 N H_2SO_4	5 - 25 %
Polysaccharides	NaOH, HCOOH, hot water	< 5%
Clay bound biochemicals	HF	5 - 50 %
"free" biochemicals (amino acids, sugars)	H_2O, 80 % alcohol, ammonium acetate	1 %
Fats, waxes, resins	Usual "fat" solvents	2 - 6 %

Tables 3, 4 and 5 show some examples of the variation in the amounts of sugars, lipids and nitrogen containing compounds in various soils.

Table 3 Monosaccharide Composition of some Soil examples and Percentage of the Organic Matter Recovered in the sugars identified [75]

Sugars	Mexico Silt Loam (mg/100 g)	Summit Silty Clay (mg/100 g)	Muck (mg/100 g)
Glucose	124	257	664
Galactose	37	77	319
Mannose	40	105	282
Xylose	36	53	222
Arabinose	39	99	326
Rhamnose and Fucose	21	56	187
Total sugars	297	647	2000
OM in soil (%)	2.1	4.4	59.0
OM as sugars (%)	14.1	14.7	3.4

Table 4 Quantity of Fats and Waxes (Ether soluble) and Resins (Alcohol soluble) Materials in various Mineral Soils (in % of humus) [76]

Soil	Fats and waxes	Resins	Total
Summit soil (Missouri), A horizon	3.56	0.58	4.14
Grassland soil (Kansas), A horizon	4.71	1.53	6.24
Grassland soil (Alberta, Canada)(1-25 cm)	0.80	0.82	1.62
Grassland soil (Manitoba, Canada)(1-20 cm)	0.46	0.84	1.30
Grassland soil (Manitoba, Canada)(25-50 cm)	0.52	0.63	1.15
Brown soil (Saskatchewan, Canada)(1-20 cm)	1.02	0.88	1.90
Prairie soil, dark-colored, A horizon	0.62	0.61	1.23
Alpine humus, Pikes Peak (13800 ft elevation)	0.94	3.10	4.04

Table 5 Distribution of the Forms of N in representative Surface Soils of the World, HUN = hydrolyzable unknown N

	Form of N (%)					
Location	Acid insoluble	NH_3	Amino acid	Amino sugar	HUN	Reference
Africa						
Sierra Leone	19.8-24.5	9.3-20.3	22.8-33.3	4.1-13.8	17.4-40.2	[48]
Tanzania	14.4-30.7	10.8-21.4	18.6-31.2	5.2-11.5	24.1-36.0	[65]
Argentina						
Misc. group	22.2-32.7	15.1-21.3	13.3-20.2	2.0-10.9	22.2-32.7	[66]
Canada						
Alberta	18.4-26.4	13.7-21.2	26.8-35.8	4.8-11.9	12.9-25.0	[67]
Quebec	16.2-33.6	15.7-28.2	23.4-37.6	4.8-9.2	6.9-22.5	[68]
Japan						
Misc. group	11.4-43.6	15.3-24.2	16.9-43.3	1.2-8.8	19.4-26.6	[69], [70]
United Kingdom						
Misc. Group	14.0-17.0	16.0-37.0	20.0-41.0	4.0-12.0	13.0-34.0	[71]
United States						
Iowa	18.4-36.7	18.6-29.0	17.8-34.3	3.3-7.1	17.9-28.9	[72]
Nebraska	18.0-22.0	18.0-28.0	31.0-46.0	5.0-9.0	6.0-17.0	[73]
West Indies						
Volcanic	11.5-41.3	11.6-17.4	25.4-45.7	0.8-3.0	4.2-35.0	[74]
Nonvolcanic	6.9-22.7	21.5-32.1	20.4-49.8	3.6-7.9	6.5-20.7	[74]

Table 6 shows some examples of the variation in the amounts of organic matter clay complexes as percentage of the total organic matter, in various soils.

Table 6 Proportion of soil organic carbon contained in the clay organic complex in some soil samples [77]

Soil	C in clay-organic complex % of total soil C
Rendzina	66.5
Pozol	89.6
Chernozem	85.2
Silt under old pasture	77.5
Brown earth	68.1
Red-brown earth	71.5
Lateritic red earth	97.8
Solodized solonetz	76.4
Solonized brown soil	51.6