The conformational structure of natural organic matter

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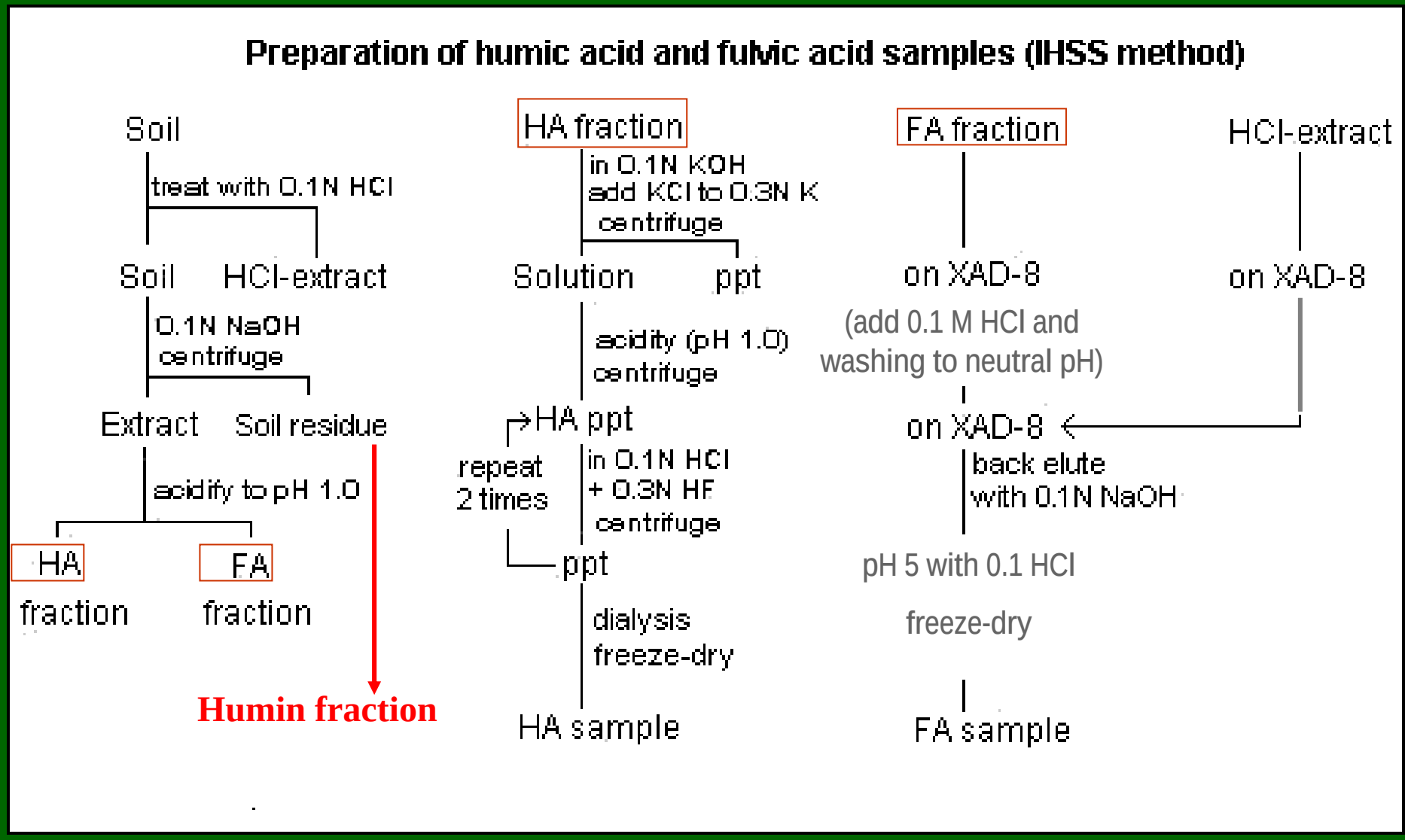
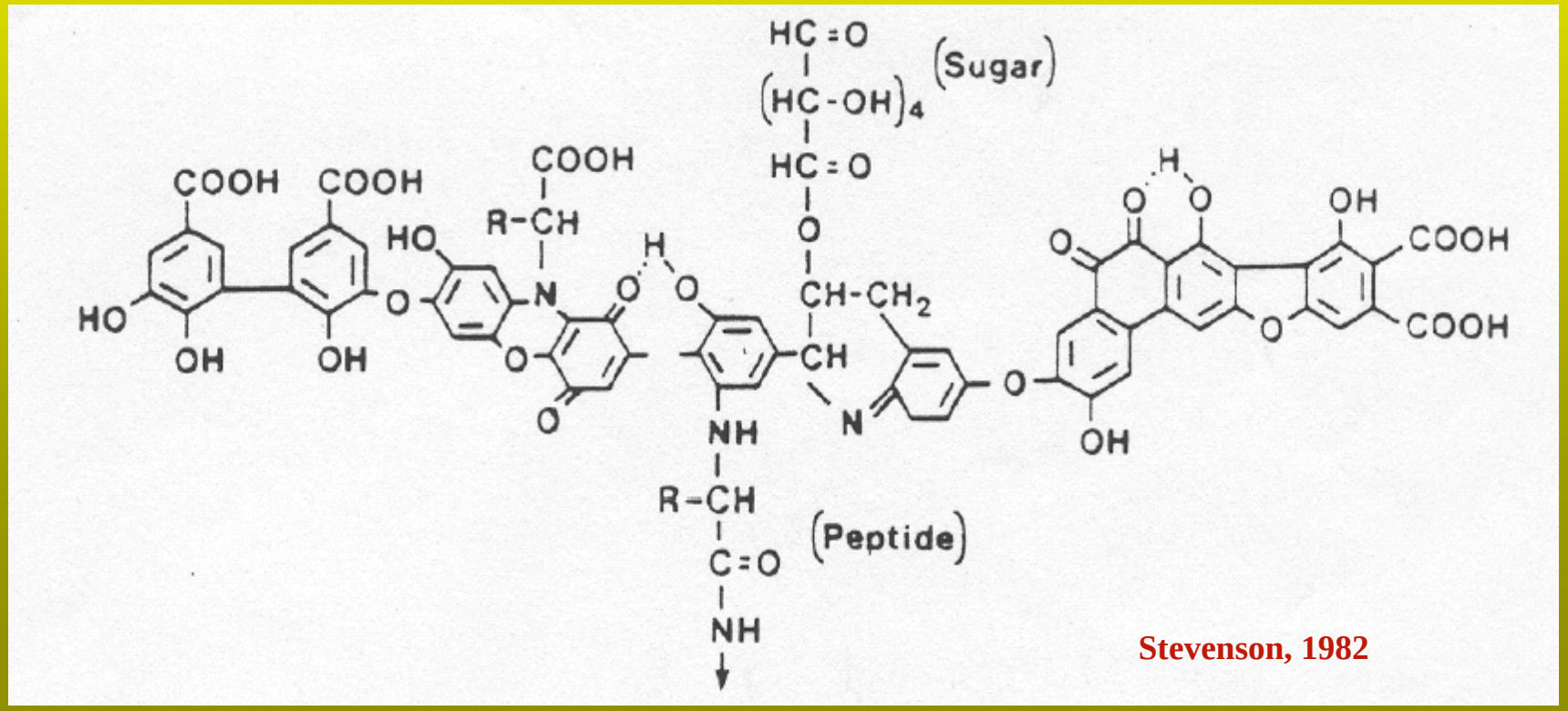
WHAT IS HUMIC MATTER?

ISOLATION AND PURIFICATION PROCEDURES TO OBTAIN HUMIC AND FULVIC ACIDS FROM SOIL

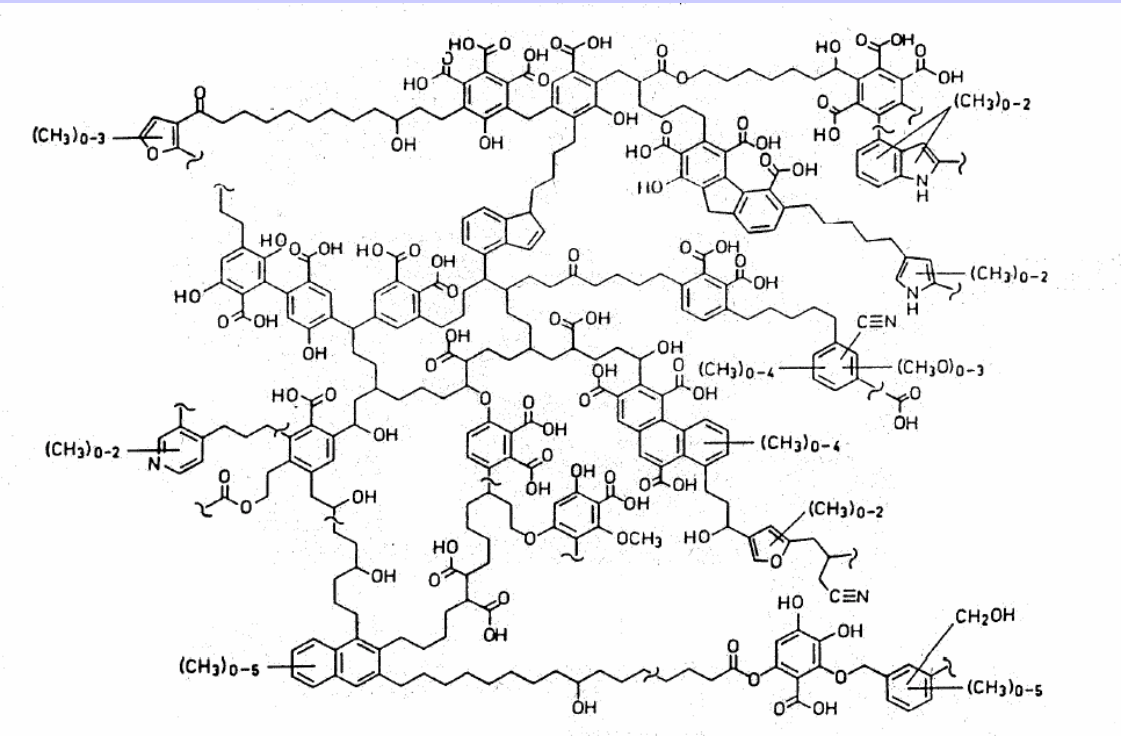


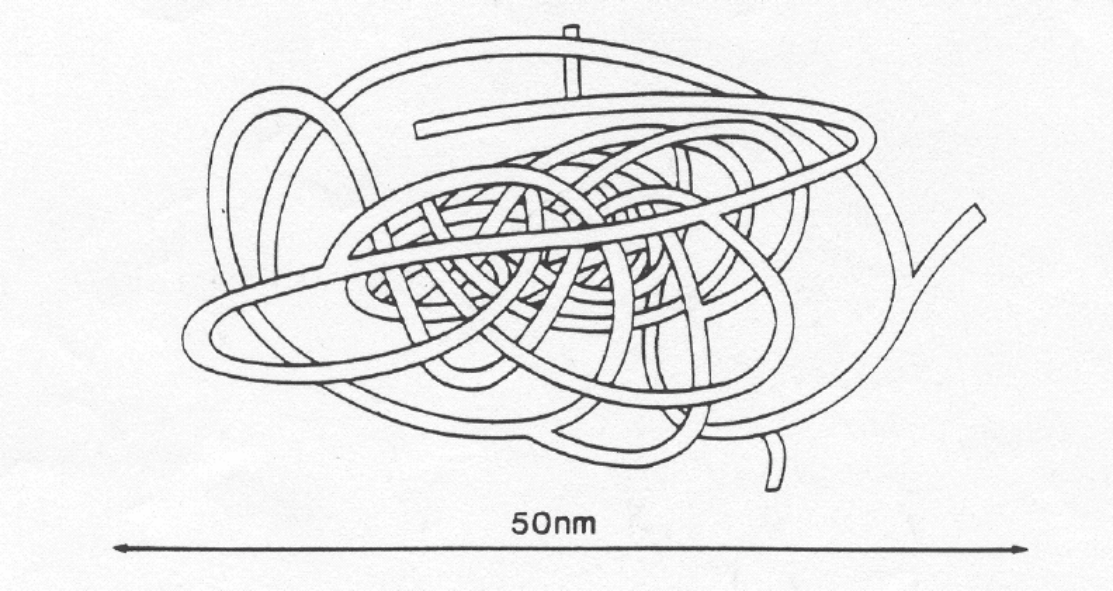
Humic substances (humic and fulvic acids) have been traditionally described as macropolymers (10-500 KDa), similarly to biopolymers, and their synthesis was attributed to an extracellular enzymatic catalysis.

The assumed monomer of such a humic macropolymer has been from time to time described with a hypothetical structure according to the updating of new experimental results obtained with progressively more advanced analytical techniques.



Stevenson, 1982

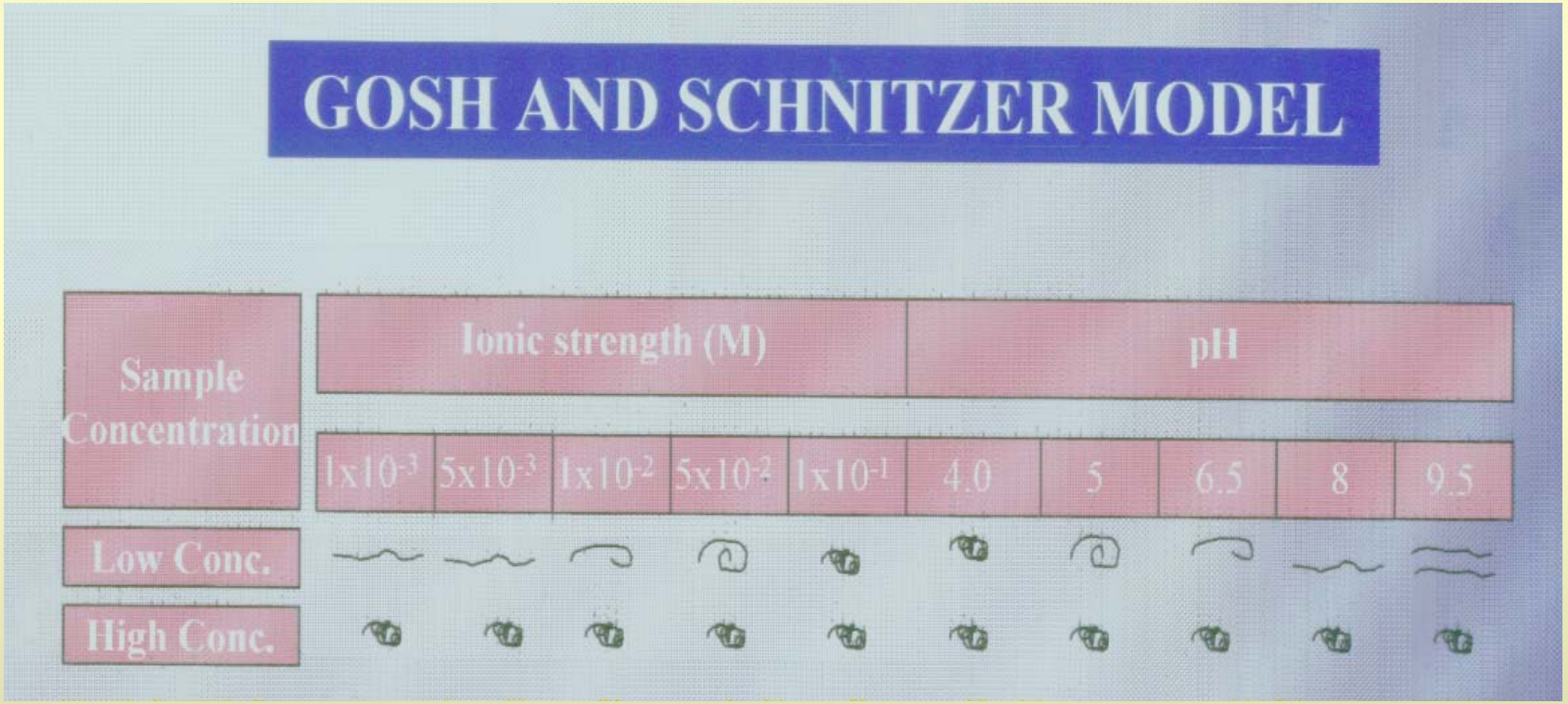




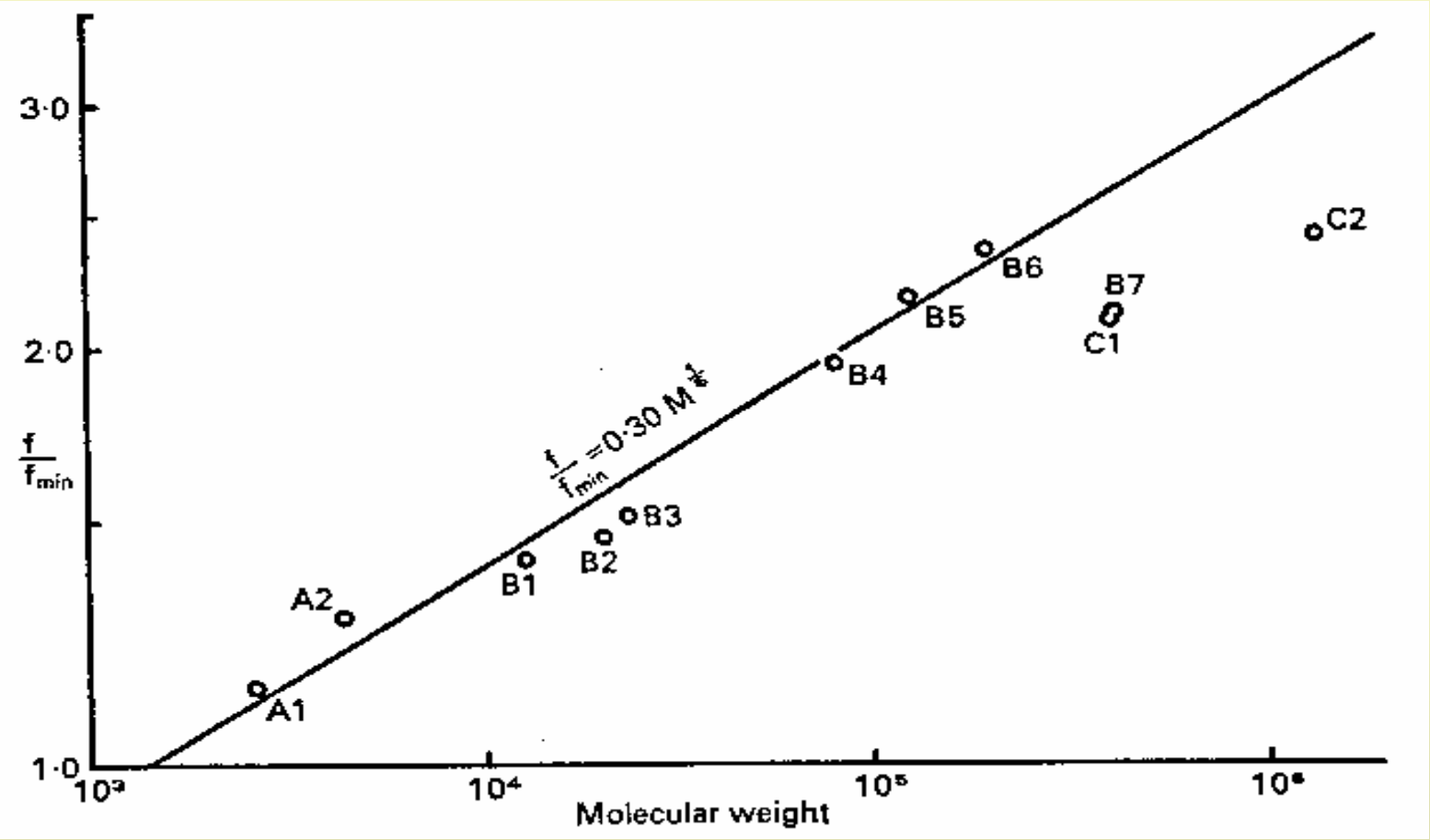
Such macropolymeric theory of humic substances became an accepted paradigm.

A number of structures, either linear or branched, but all stabilized by covalent bonds, have been proposed and became common in textbooks and scientific reports.

By applying viscosity and and surface measurements to the Flory and Fox equation derived to describe macropolymers, Gosh and Schnitzer (1980) depicted humic matter as linear polymers having size and shapes depending on ionic strength and pH of solution and concentration of sample.



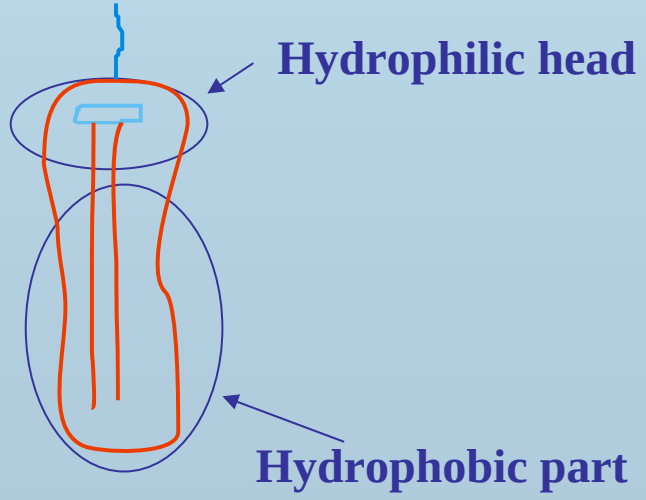
Assuming humic matter to be a polydisperse macropolymeric mixture, Cameron et al. (1972) employed ultracentifugation by sedimentation velocity technique, to determine the MW of a number of fractions of HA separated by Gel Permeation Chromatography. The MW values were reported to range from 10^2 to 10^6 Dalton

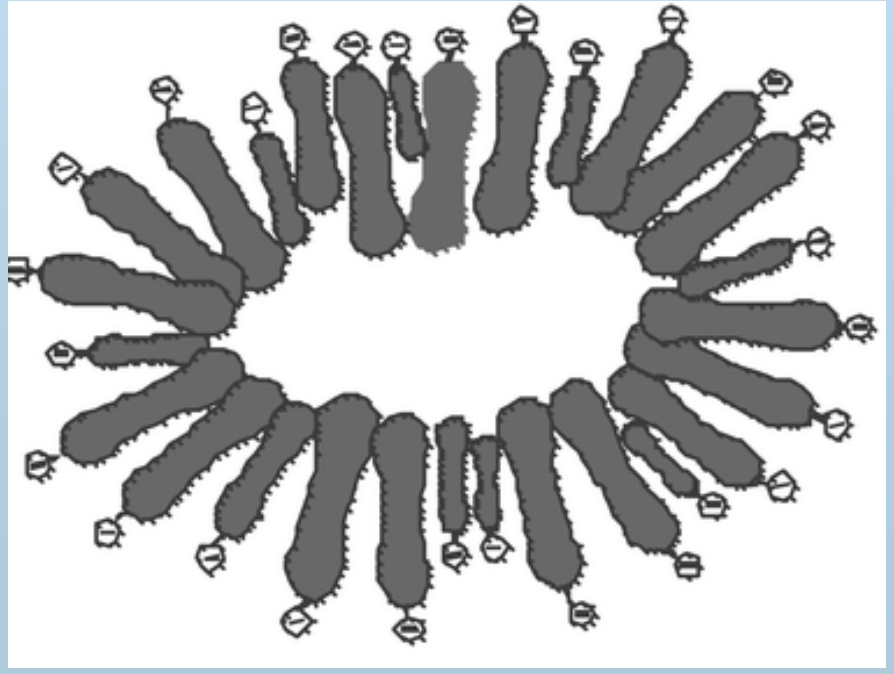


WERSHAW's MODEL

To explain the evident surfactant properties of humic substances, and their capacity to solvate hydrophobic contaminants, Wershaw (1994) proposed that humic substances be aggregates or micelles of amphiphilic molecules with polar and apolar tails as much as in cell membrane arrangements.

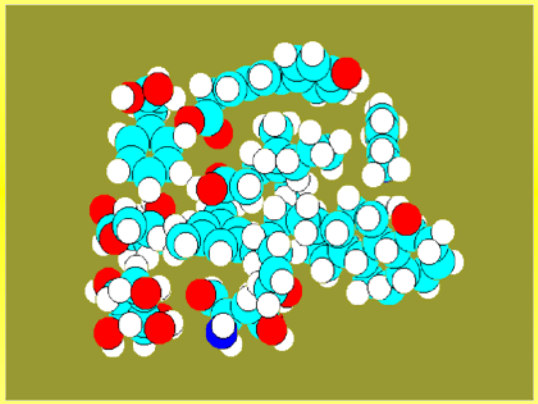
However, he did not directly approched the molecular size of the aggregating molecules.



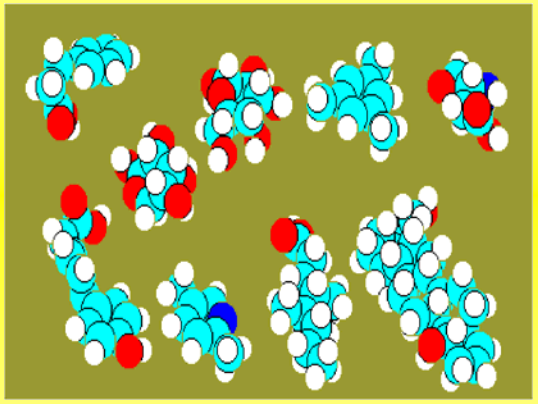


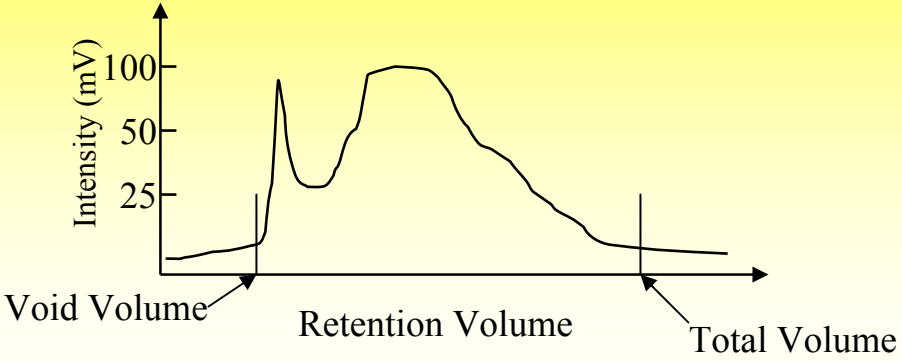
THE SUPRAMOLECOLAR STRUCTURE

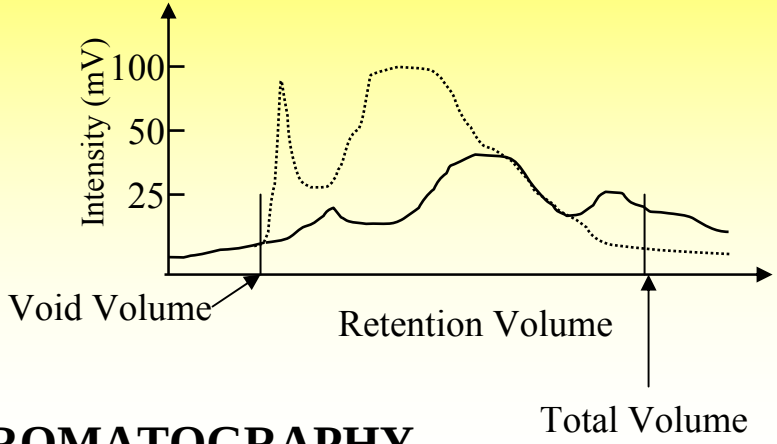
Experimental results (HPSEC, NMR, ESI-MS) indicate that humic substances, rather than being macropolymers, are supramolecular self-associations of heterogeneous and relatively "small" molecules stabilized by weak bonds (H-bonds, van der Waals, π-π, CH-π), which may be disrupted by small amounts of organic acids.



CH_3COOH →







SIZE-EXCLUSION CHROMATOGRAPHY

PRINCIPLES OF SIZE-EXCLUSION CHROMATOGRAPHY

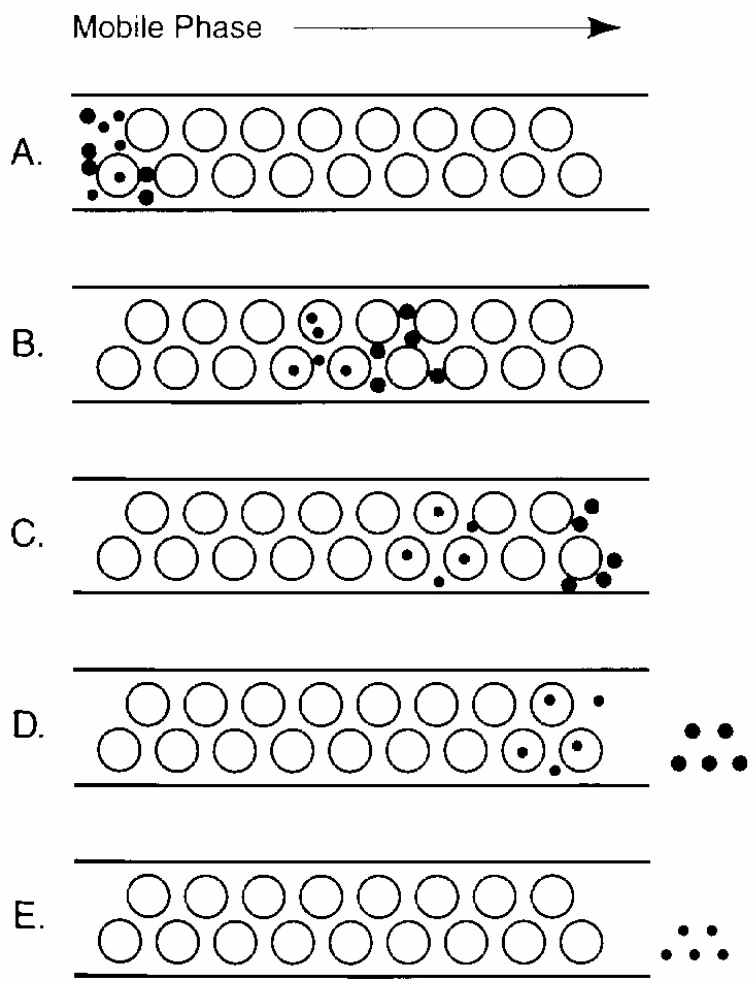


Fig. 2.1. Schematic of an SEC separation showing the separation of low (•) and high MW (●) polymers: *A* start of separation; *B* smaller molecules diffuse into porous particles, while larger molecules elute in the interstitial regions of the packed bed; *C* size separation is completed; *D* large molecules, which sample less column volume, elute first; *E* small molecules having access to both interstitial and pore volumes elute later

MOLECULAR DIMENSION AND ELUTION VOLUME

Small molecular dimension

Intermediate

Large molecular dimension

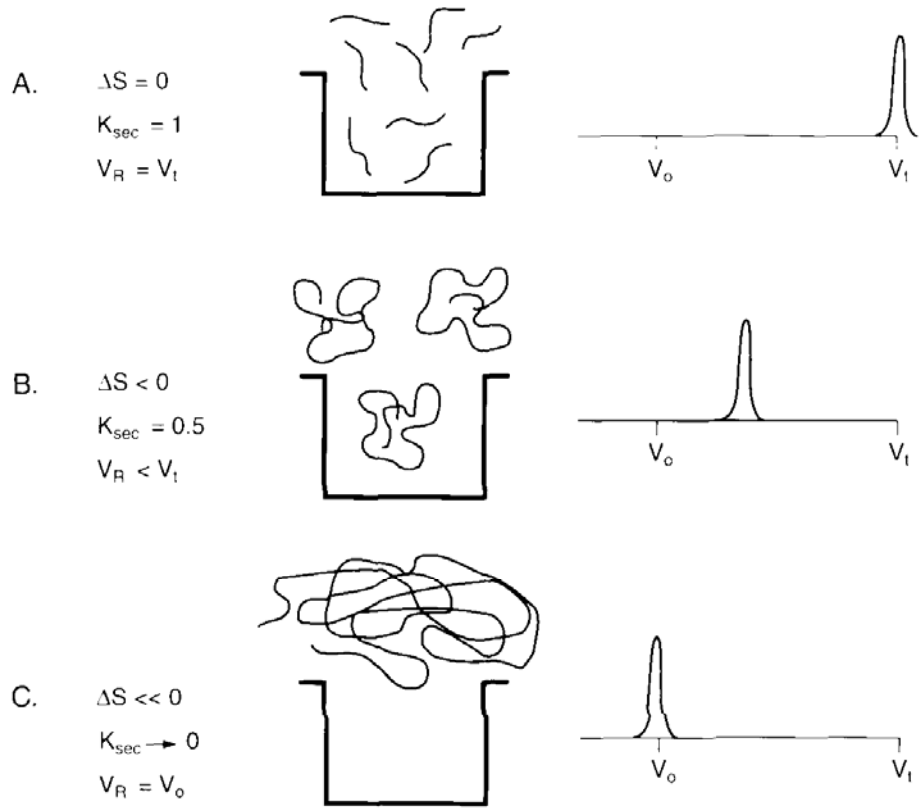
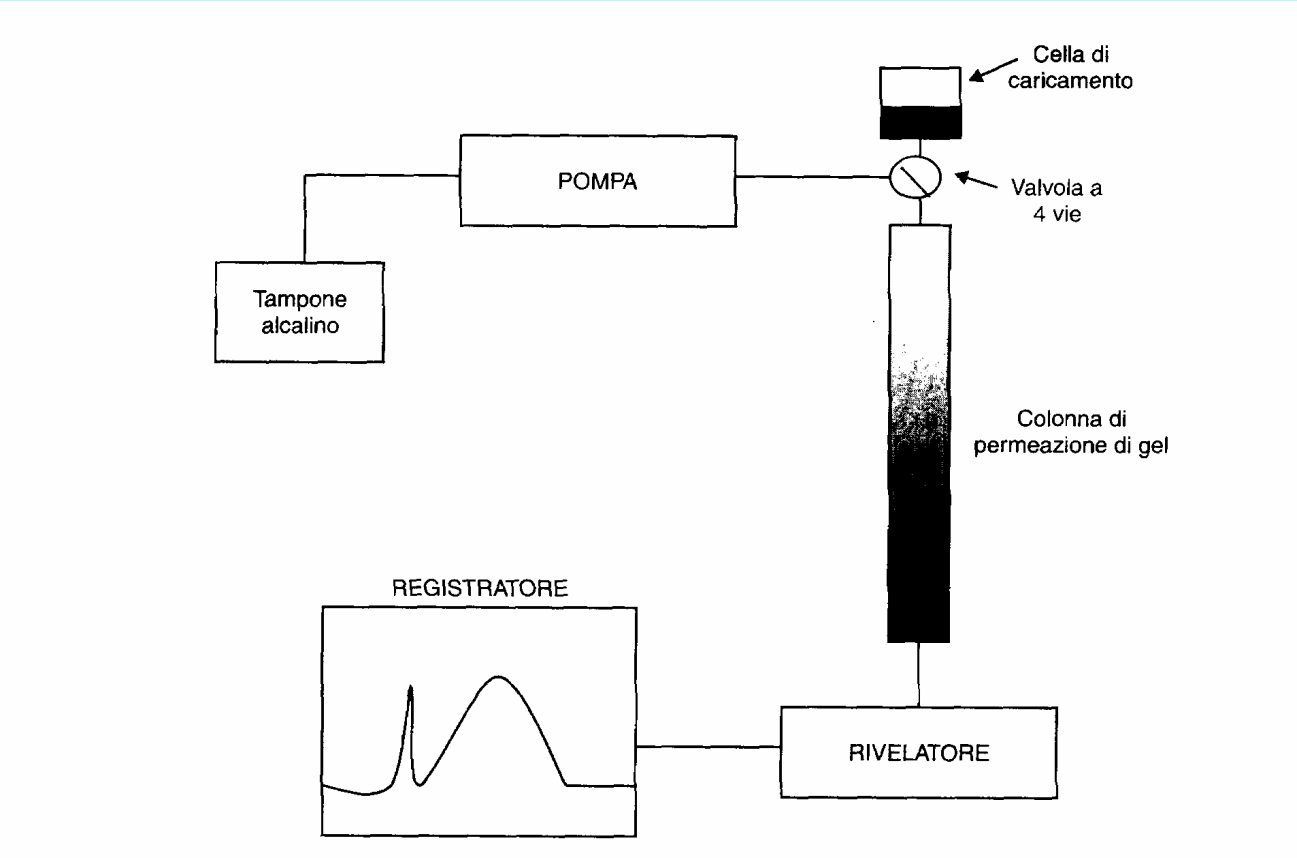


Fig. 2.5. Thermodynamic representation of the SEC process. Elution of: *A* totally permeated molecules; *B* partially excluded molecules; *C* totally excluded molecules

SCHEMATIC REPRESENTATION OF A SIZE-EXCLUSION CHROMATOGRAPHY SET-UP



GEL PERMEATION IN $Na_4B_2O_7$ pH 9.2

0.02M 0.1M

Low pressure

A. HA dissolved at pH 9.2

B. Titrated to pH 6.0 with acetic acid

C. to pH 4.5 with acetic acid

D. to pH 3.5 with acetic acid

E. to pH 2.0 with acetic acid

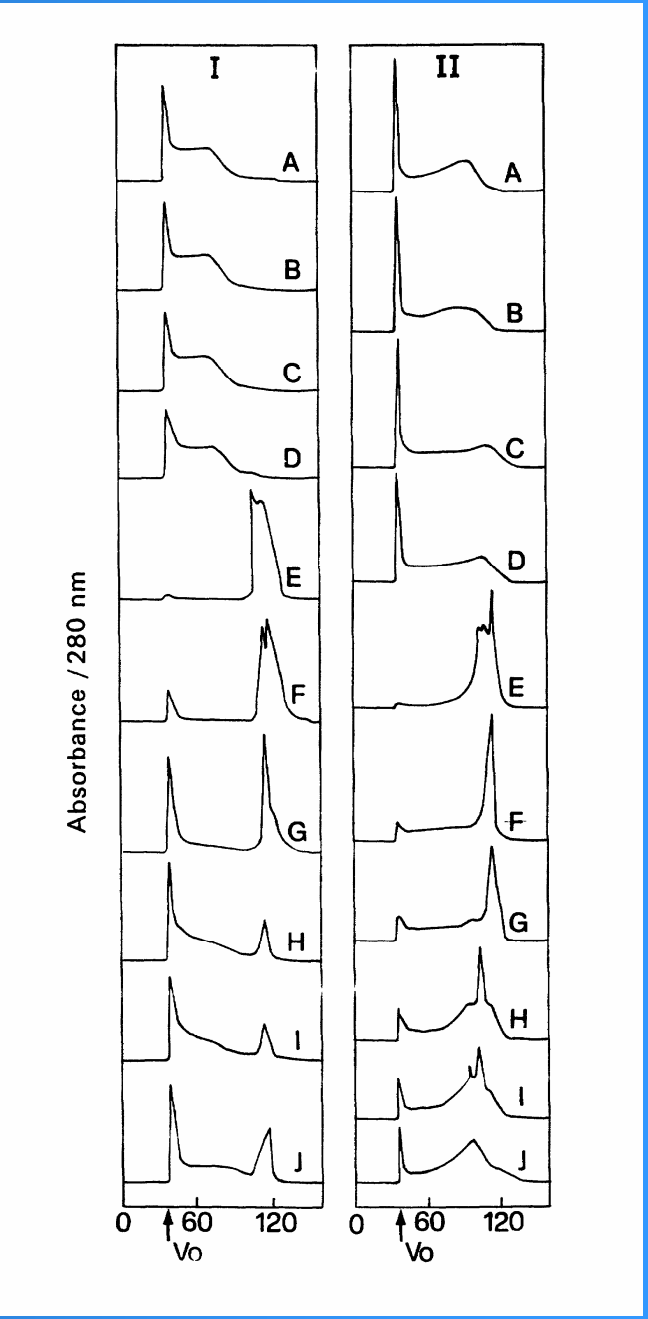
F. Backtitrated to pH 3.5 with KOH

G. to pH 4.5 with KOH

H. to pH 6.0 with KOH

I. to pH 8.5 with KOH

J. and rotoevaporated at pH 8.5



Piccolo, Nardi, Concheri (1996). European Journal of Soil Science, 47:319-328

HPSEC elution in $NaNO_3$ 0.05 M pH 7

Monocarboxylic acids

A= HA (volcanic soil) pH 7
***Mw*=32961**

B= to pH 3.5 with HCl

Δ*Mw*=-13.7%

C=with HCOOH

Δ*Mw*=-20.8%

D=with CH_3COOH

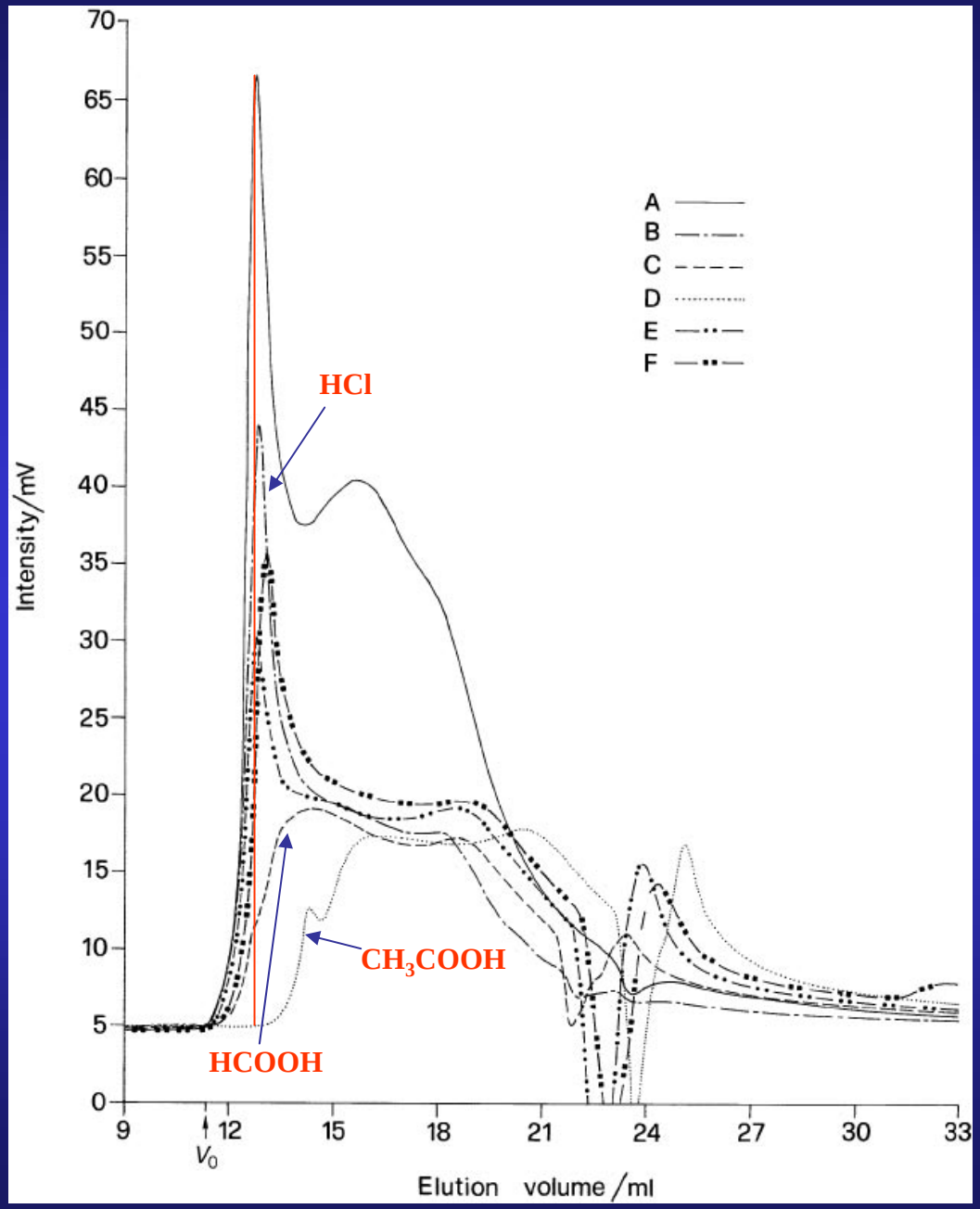
Δ*Mw*=-29.8%

E=with CH_3CH_2COOH

Δ*Mw*=-5.7%

F= with $CH_3(CH_2)_2COOH$

Δ*Mw*=+2.3%



Piccolo, Conte, Cozzolino (1999). *European Journal of Soil Science*, 50:687-694

HPSEC elution in $NaNO_3$ 0.05 M pH 7

Sulphonic acids

A= HA (oxidized coal) pH 7

Mw=9418

B=to pH 3.5 with HCl

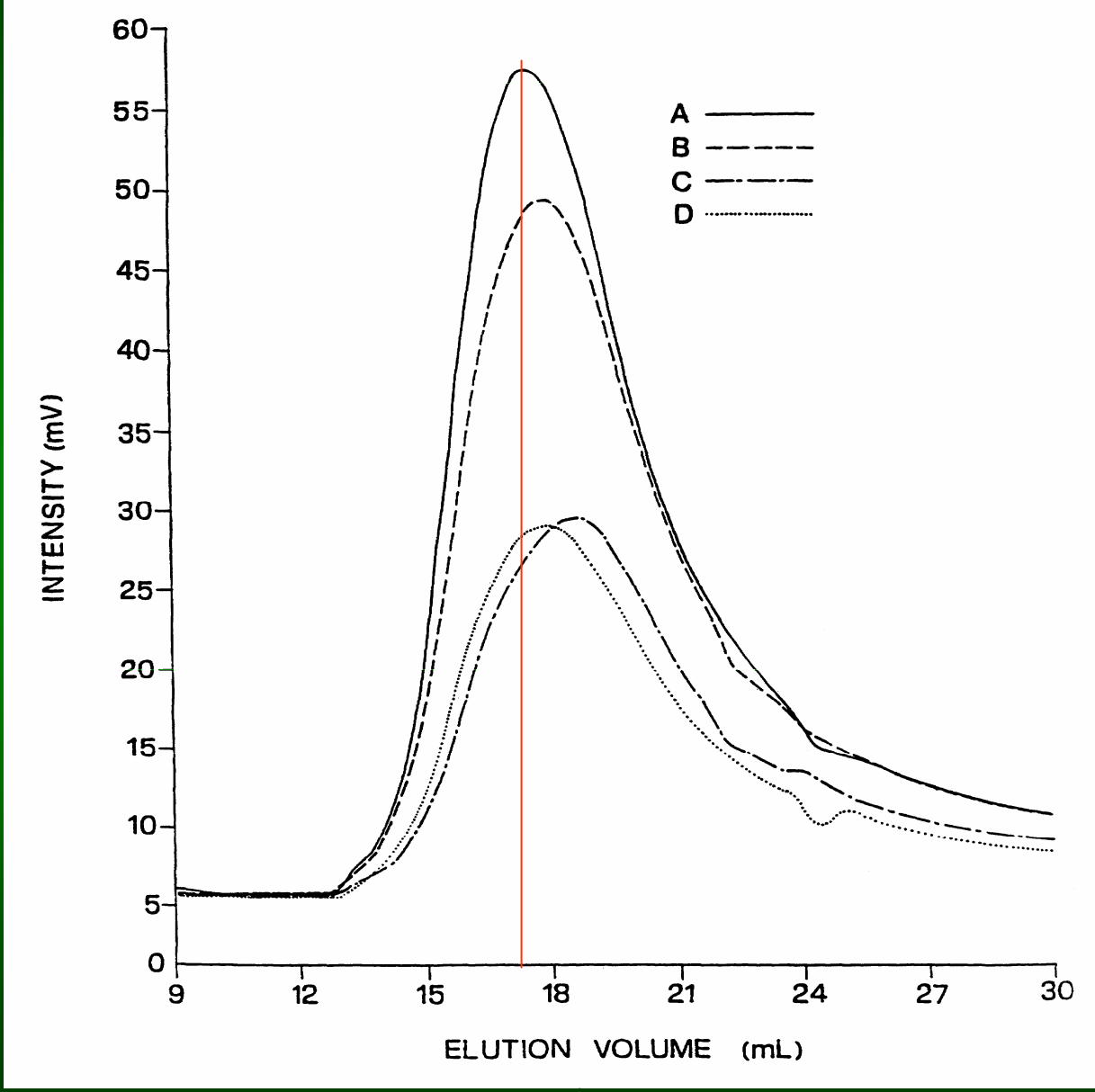
Δ*Mw*=-8.4%

C= to pH 3.5 with methansulphonic acid,

Δ*Mw*=-27.0%

D= to pH 3.5 with benzensulphonic acid

Δ*Mw*=-28.7%



Cozzolino, Conte,Piccolo (2001) *Soil Biology and Biochemistry*, 33:563-571

HPSEC elution in $NaNO_3$ 0.05 M pH 7

Dicarboxylic acids

A=HA (oxidized coal) pH 7

B= to pH 3.5 with HCl

ΔMw=-14.4 %

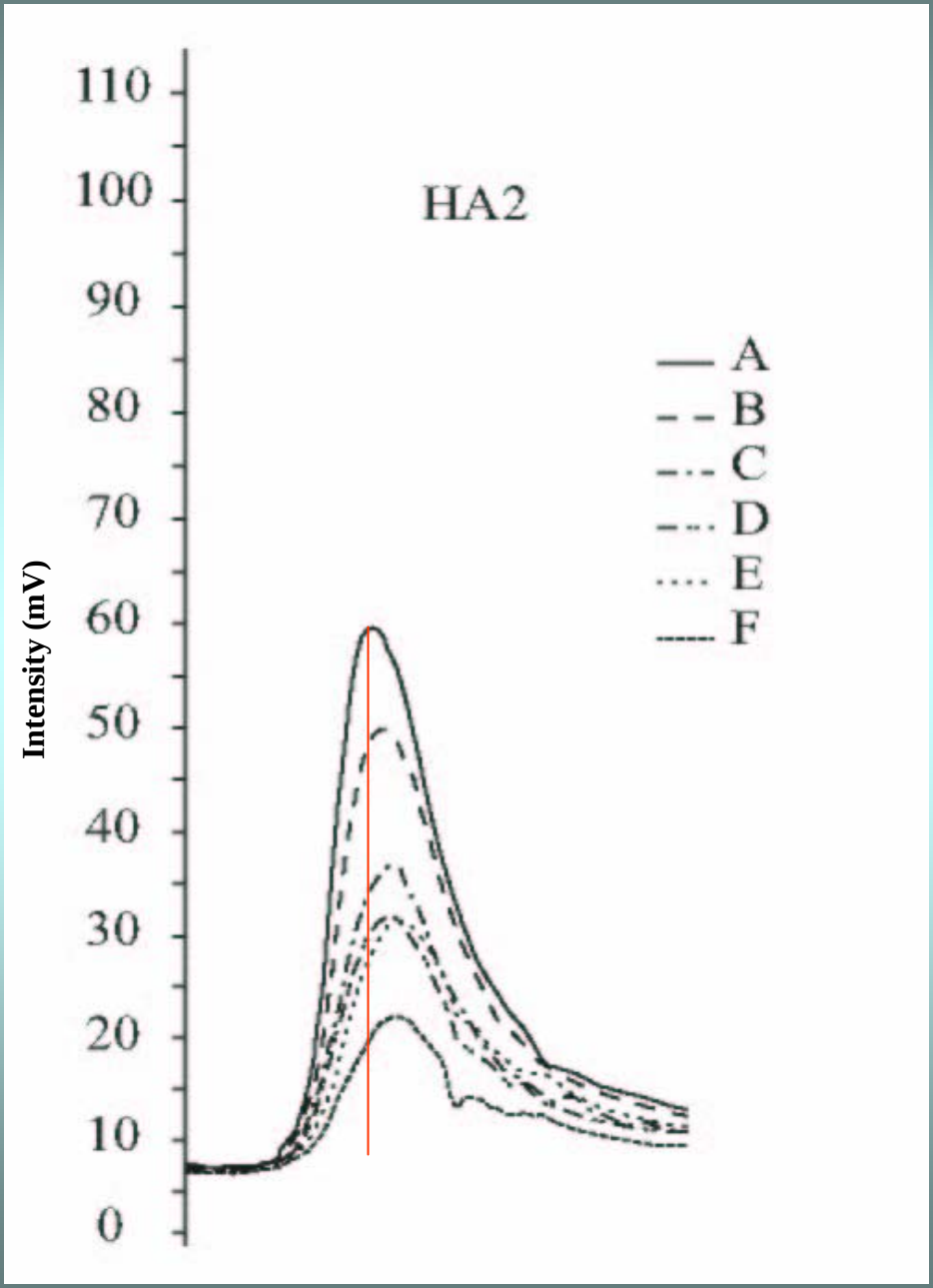
C=with HOOC-COOH ,
ΔMw=-41.7 %

D=with HOOC-CH_2-COOH
ΔMw=-48.2 %

E=with HOOC-$(CH_2)_2$-COOH ,

ΔMw=-50.0 %

F=with HOOC-$(CH_2)_3$-COOH
ΔMw=-72.2 %



Piccolo, Conte, Spaccini, Chiarella (2003). *Biology and Fertility of Soils*, 37:255-259

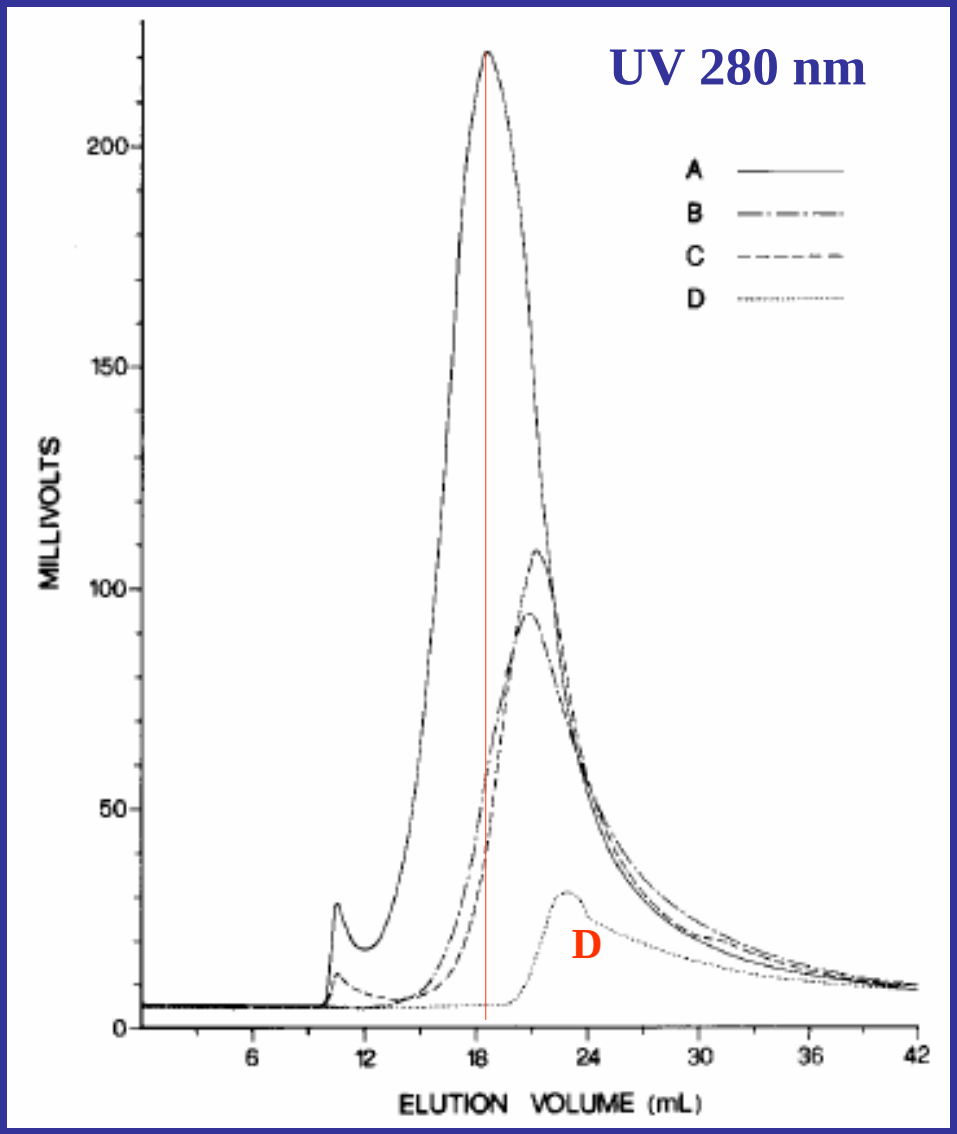
EFFECT OF ELUENT COMPOSITION ON MOLECULAR SIZE DISTRIBUTION OF HA

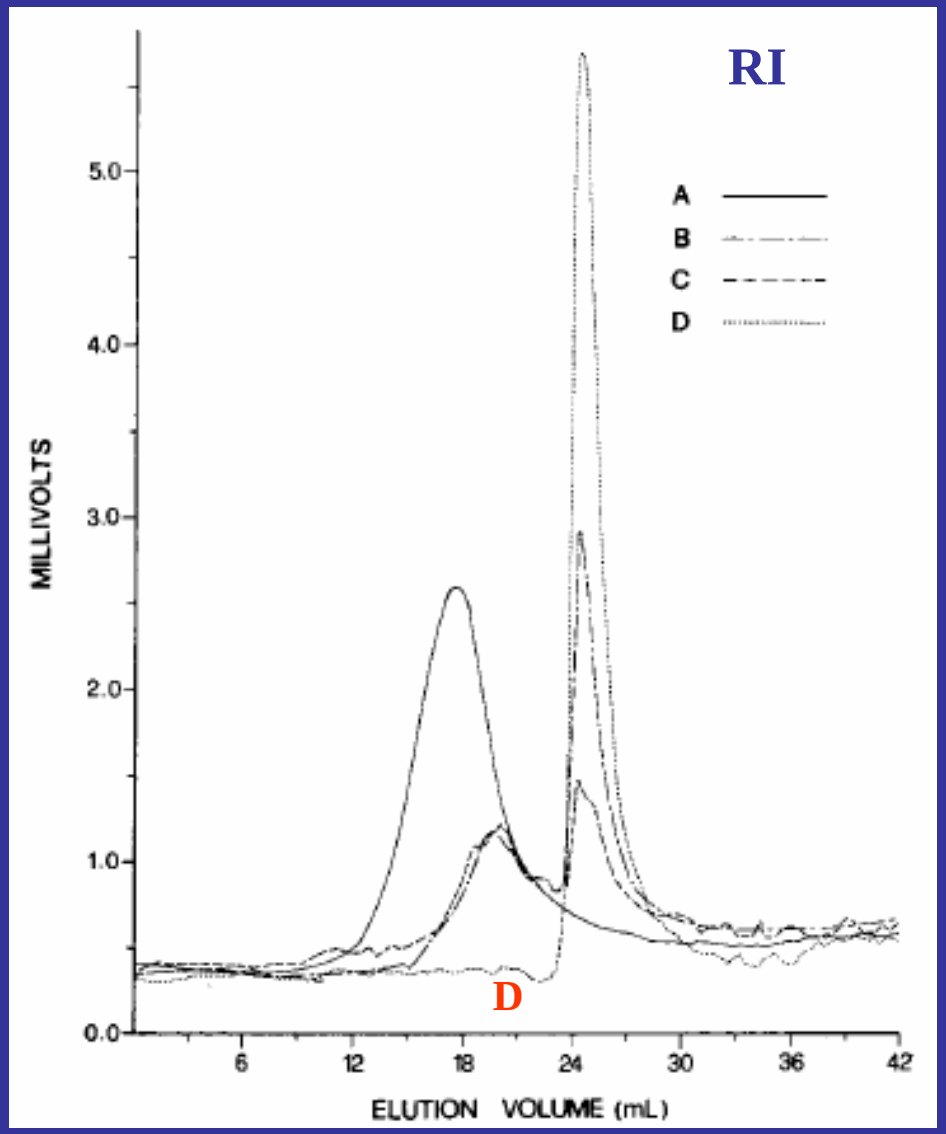
INSTEAD OF LOWERING THE pH OF HUMIC SOLUTIONS, WERE THE MOBILE PHASES TO BE VARIED IN pH BUT NOT IN IONIC STRENGTH

SOLUTIONS OF HUMIC SUBSTANCES DISSOLVED WITH CONTROL ELUENT AT pH 7 WERE INJECTED INTO HPSEC AND ELUTED WITH MOBILE PHASE MODIFIED BY ADDITION WITH METHANOL (pH 6.97), HCl (pH 5.54) AND ACETIC ACID (pH 5.7)

HA (Lignite) in different HPSEC eluents

A= $NaNO_3$ pH 7; B= +CH_3OH pH 6.9; C=+HCl pH 5.54; D=+CH_3COOH pH 5.7





Conte, Piccolo. *Environmental Science & Technology* (1999) 33:1682-1690

HPSEC BEHAVIOUR

HUMIC SUBSTANCES VERSUS UNDISPUTED POLYMERS

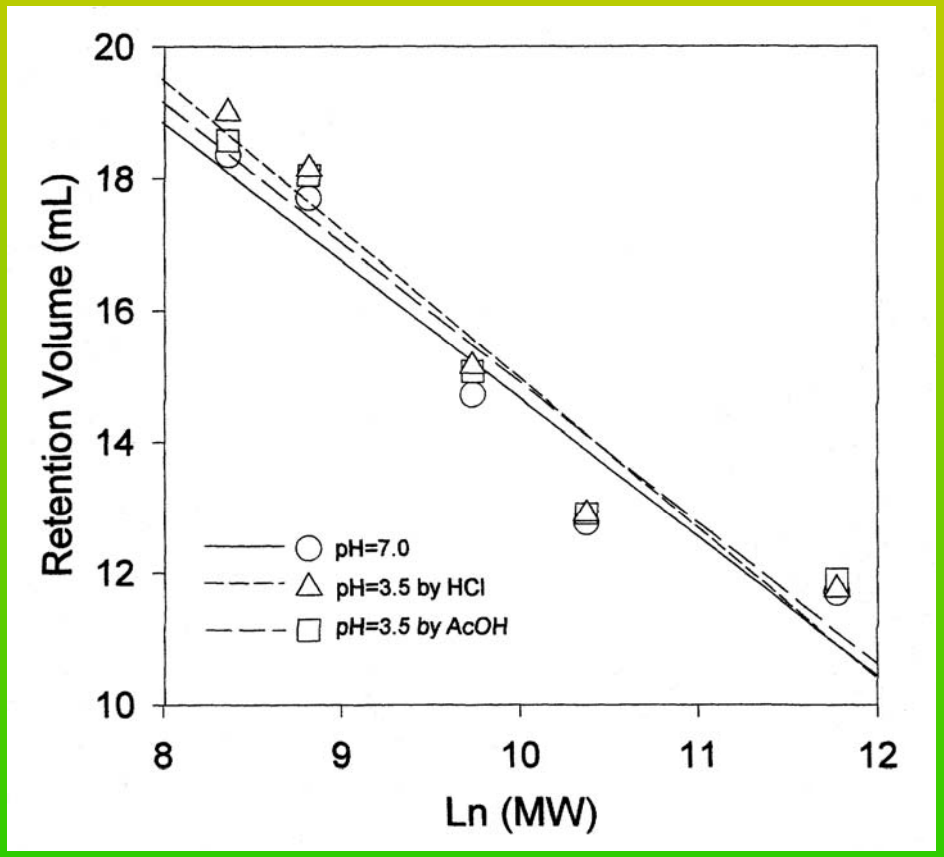
Polymers such as polysaccharides and polystyrenesulphonates are commonly used as proxy standards to evaluate nominal molecular sizes of humic substances

However, if humic substances are supramolecular associations, they should have a different HPSEC behaviour than real polymers such as polysaccharides and polystyrenesulphonates

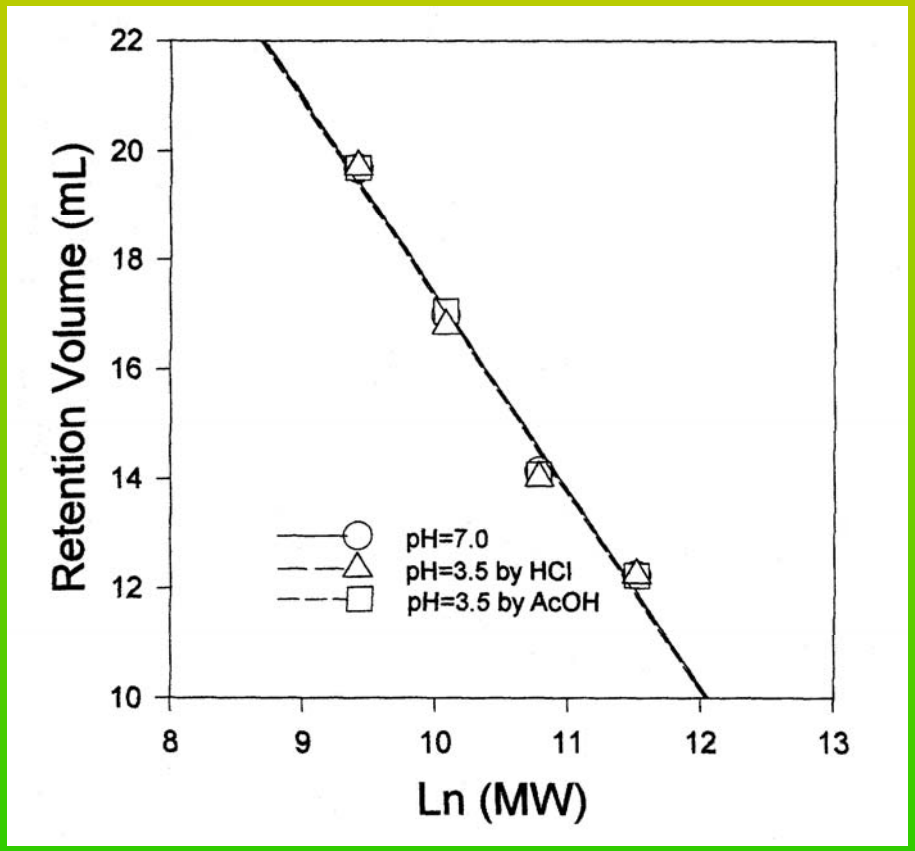
The two classes of compounds were compared when aqueous solutions were modified by addition of either HCl or acetic acid

HPSEC response to pH changes in solutions of true polymeric standards such as polystirenesulphonates (UV) and polysaccharides (Refractive Index)

UV detection



Refractive index detection



Piccolo et al. (2000). *Royal Society of Chemistry*, Special Publication no. 259, Cambridge, UK, pp.111-124

HPSEC response of true polymeric standards eluted by four different modifications of eluents:

A= $NaNO_3$ pH 7

B= +CH_3OH pH 6.9

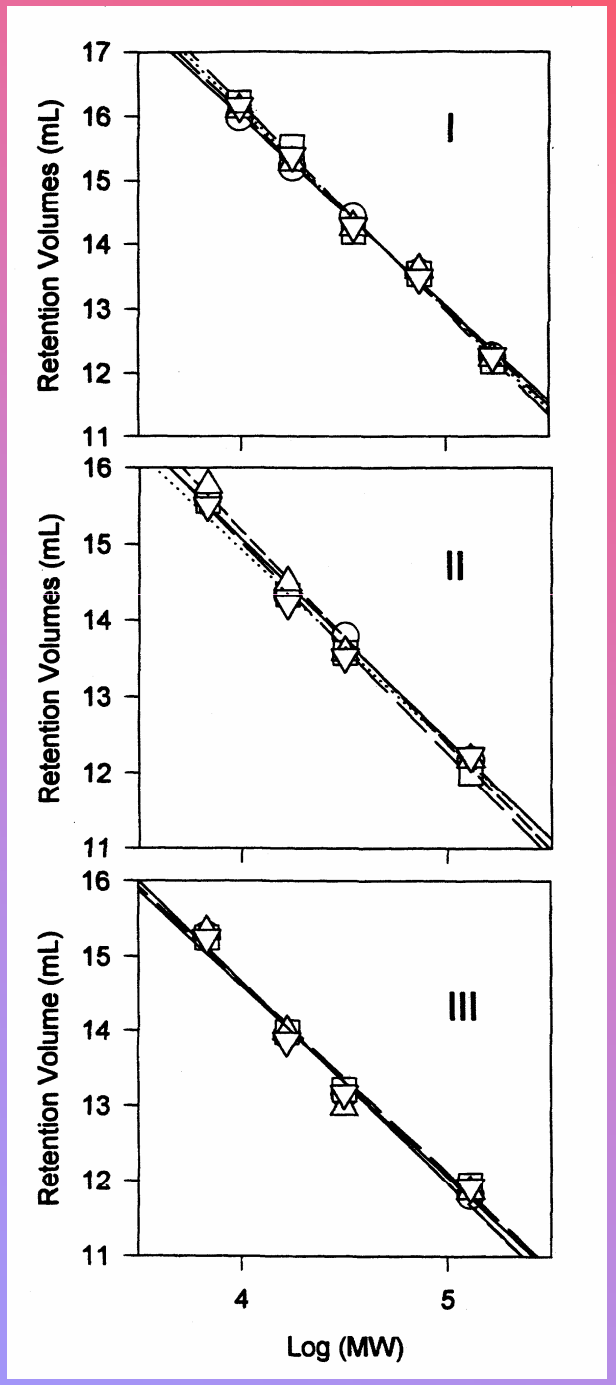
C=+HCl pH 5.54

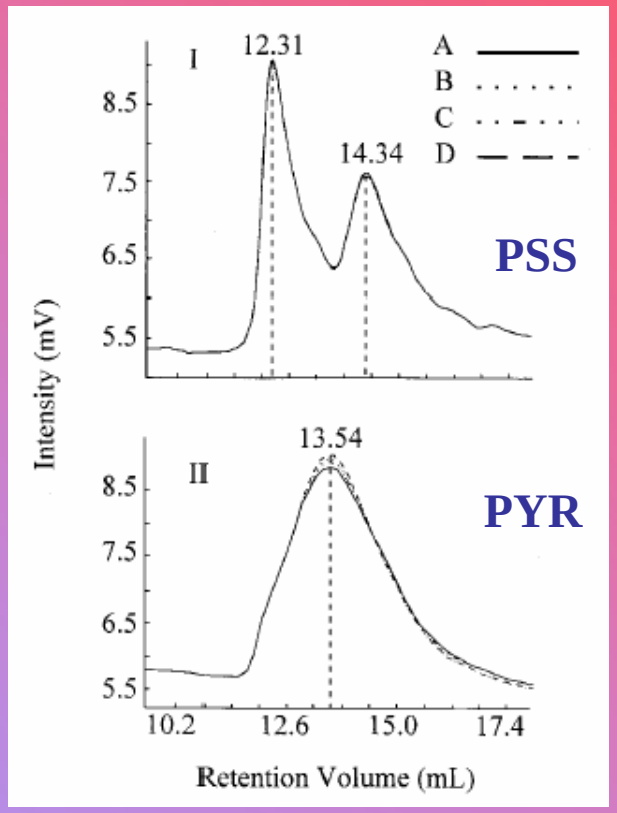
D=+CH_3COOH pH 5.7

Polysaccharides standards detected by RI

Polystirenesulphonates standards detected by UV

Polystirenesulphonates standards detected by RI





Piccolo et al.. (2001) *Soil Science*,166:174-185

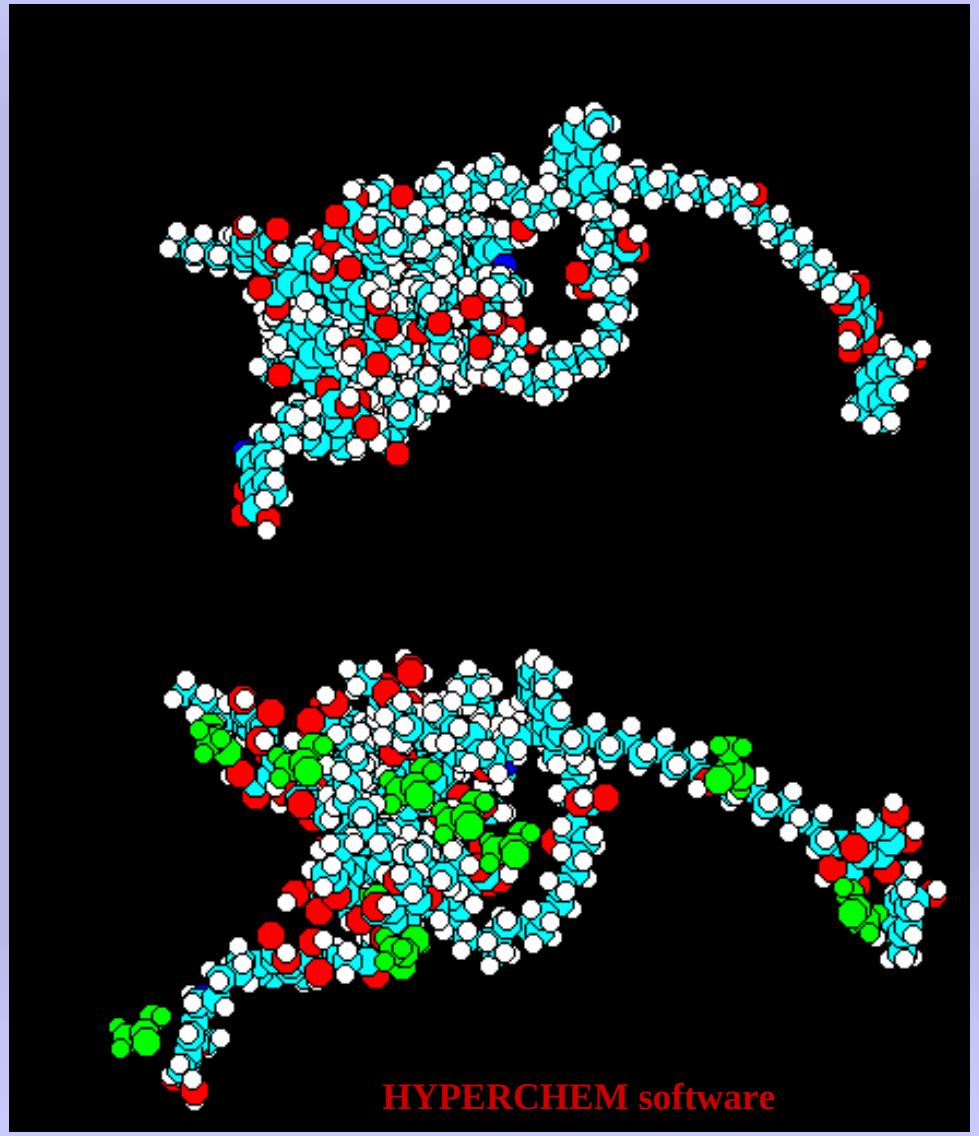
These results suggest that humic substances are supramolecular associations of heterogeneous and molecules held together by weak bonds (H-bonds, π-π, CH-π). Thermodynamic reasons force the random self-assembled molecules in only apparently large molecular sizes.

The disruption of supramolecular associations by low amounts organic acids is due to the formation of H-bonds with complementary humic sites. These H-bonds are stronger than the hydrophobic bonds stabilizing humic conformations at neutral and alkaline pHs.

Humic conformations in solution depend on both the acidity and hydrophobicity of added organic compounds.

MINIMIZATION OF CONFORMATIONAL ENERGY

POLYMERIC-COVALENT MODEL



Traditional humic macropolymeric model
MW=6326 Da

Energy = 627.4 Kcal.mol^{-1}

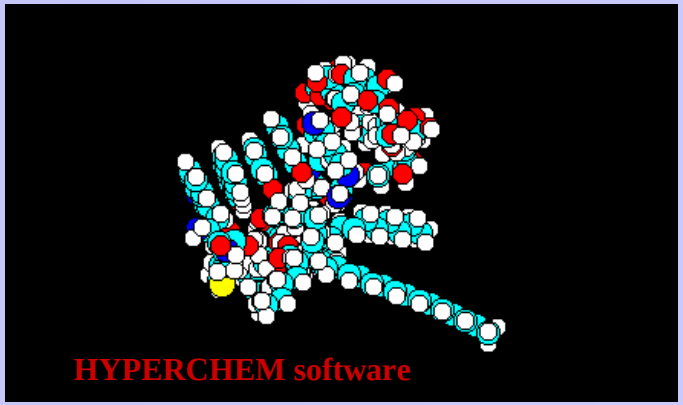
Conformational variation induced by the adsorption of 10 molecules of acetic acid

Energy = 617.26 Kcal.mol^{-1}

Gain in conformational energy = 1.6 %

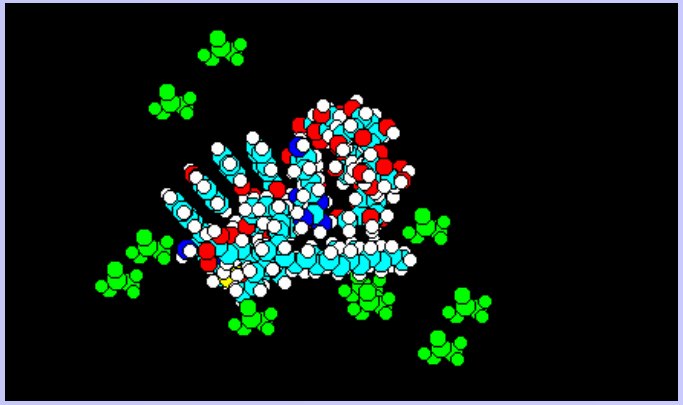
PICCOLO et al. In: D.K. Benbi and R. Nieder, Editors. *Handbook of processes and modelling in soil-plant system*. Haworth Press, New York, pp. 83-120 (2003).

MINIMIZATION OF CONFORMATIONAL ENERGY
SUPRAMOLECOLAR MODEL



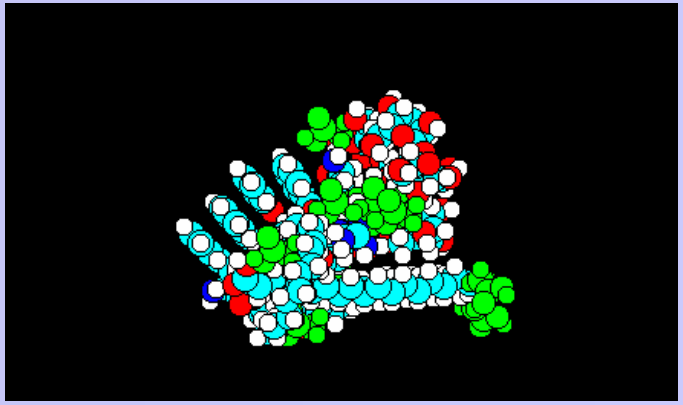
Supramolecolar Humic Model. Association of different small molecules (aminoacid, glucose, fatty acids, cinnamic acids)
total MW = 6065 Da
Energy = 114.0 Kcal.mol^{-1}

Gain in energy = 20.0 %



Conformational variation induced by the approach of 10 molecules of acetic acid
Energy= 91.2 Kcal.mol^{-1}

Gain in energy = 26.3 %



Conformational variation induced by the adsorption of 10 molecules of acetic acid
Energy= 84.0 Kcal.mol^{-1}

PICCOLO et al. In: D.K. Benbi and R. Nieder, Editors. *Handbook of processes and modelling in soil-plant system*. Haworth Press, New York, pp. 83-120 (2003).

WHAT IS THE MASS OF HUMIC MOLECULES?

The evaluation of absolute molecular masses by ESI-MS

Electrospray ionization (ESI) is a soft technique to obtain unfragmented and single-charged ions of humic molecules in aqueous solution for compatible measurements by mass-spectrometry

Humic solutions are conveyed through a capillary to the tip of a cone where are ionized and released in a droplet. The droplets are progressively size-reduced by temperature until the ions repel each other and enter separated into the mass analyzer in vacuum

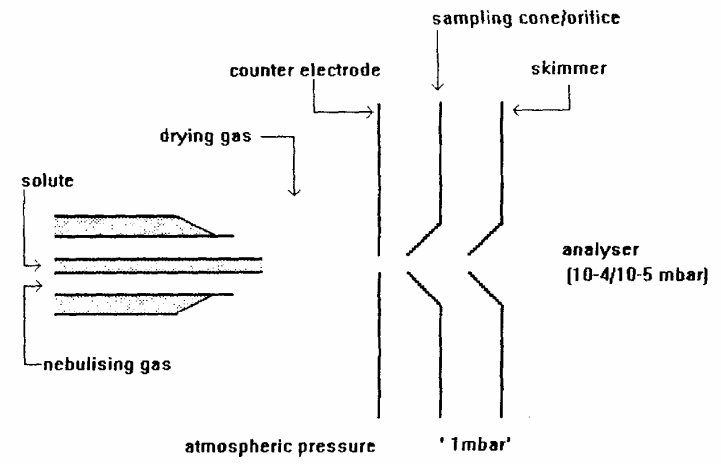


Figure 2.1 *Schematic diagram of an electrospray ionization source*

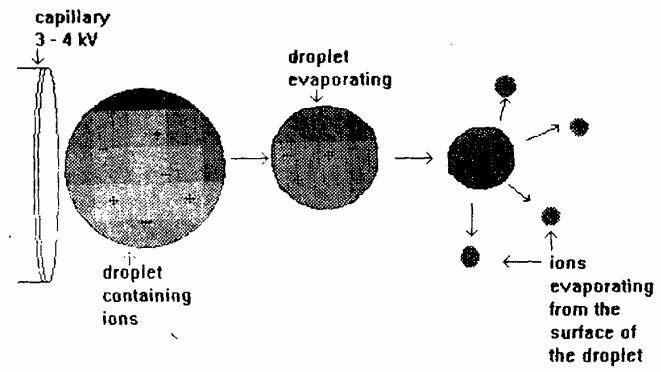


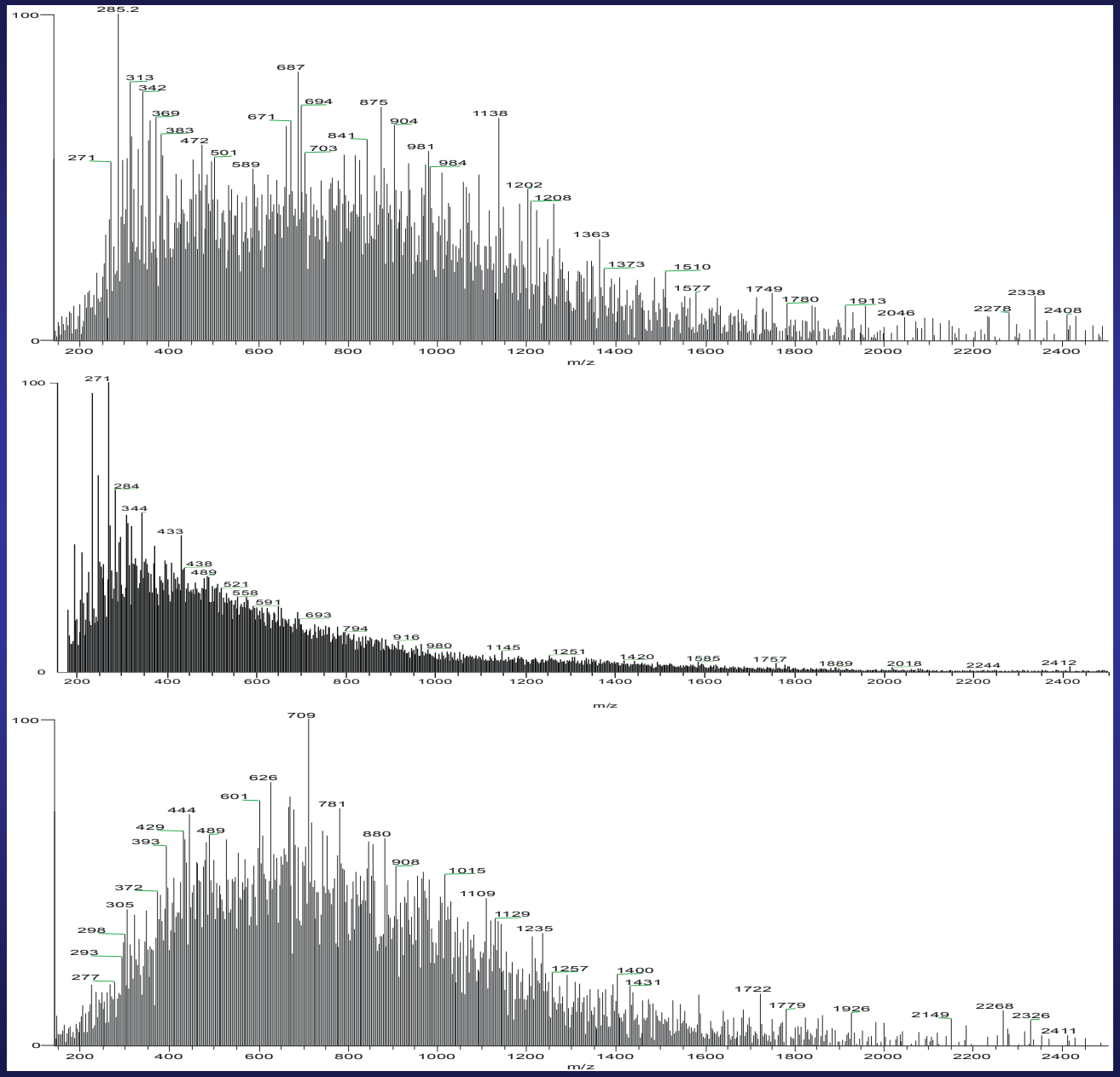
Figure 2.2 *The electrospray ionization process*

ESI mass spectra (negative) of ammonium humates and fulvates

Volcanic HA
***Mw* < 2500 Da**

Volcanic FA
***Mw* < 1100 Da**

Lignite HA
***Mw* < 2400 Da**

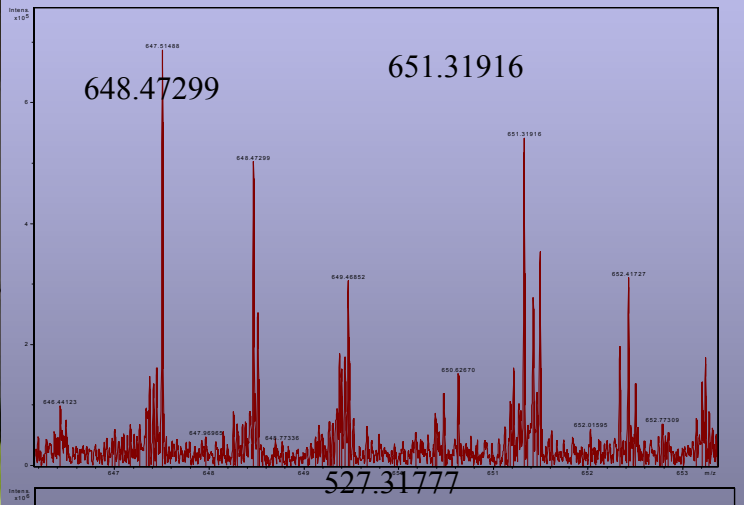


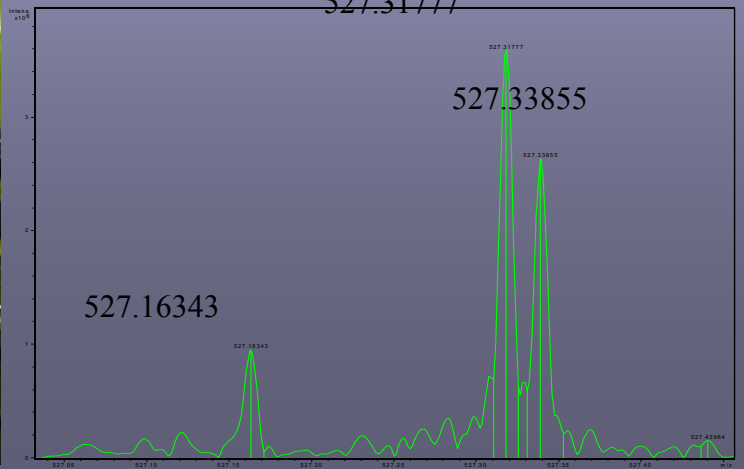
Piccolo and Spiteller (2003). *Analytical and Bioanalytical Chemistry*, 377: 1047-1059

By new advanced high-resolution Mass Spectrometers (FT IRC-MS), it was possible to ascertain not only that humic molecules are singly-charged and have low molecular mass (≤3000 Da), but also that aquatic humic substances contain up to 6.000 different molecular masses, while terrestrial humic sustances may contain more than 10.000 different masses.

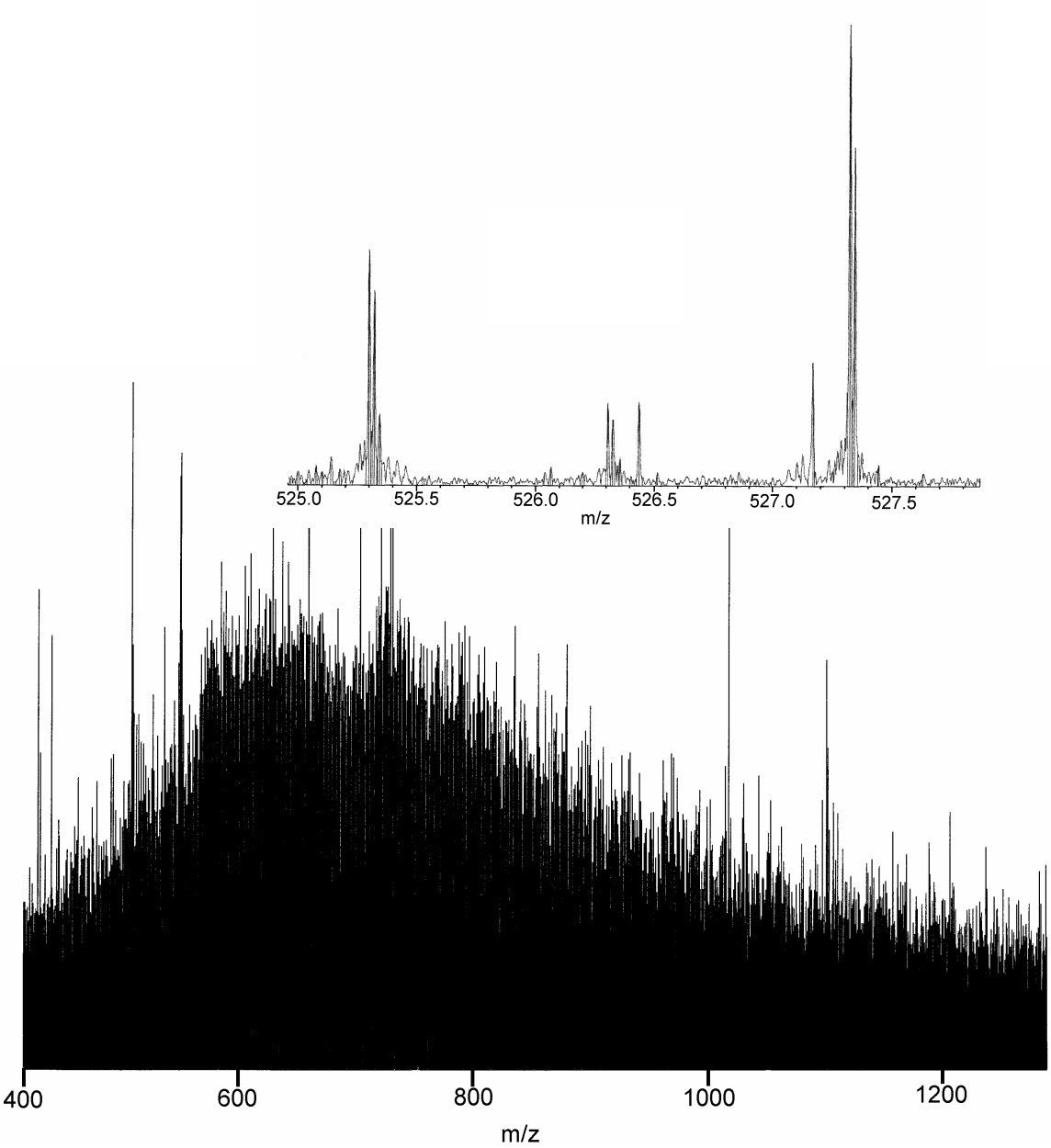
Fourier Transform Ion Resonance Cyclotron Mass Spectrometry (FT IRC-MS) with a 11.7 Tesla Magnet

Bruker Daltonics, Bremen, D





Advanced High-Resolution Mass Spectrometers (FT-IRC-MS),



High resolution mass-spectrometry excluded occurrence of multiple charges and fragmentation during ESI-MS of humic matter.

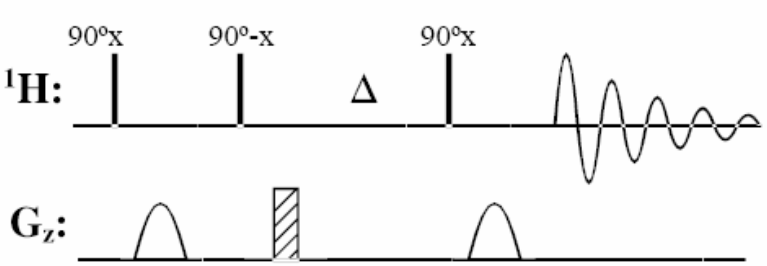
(Stenson et al., *Analytical Chemistry*. 2002, 74:4397-09)

No molecular mass larger than 1500 Da was noted by FT-IRC-MS for a terrestrial HA

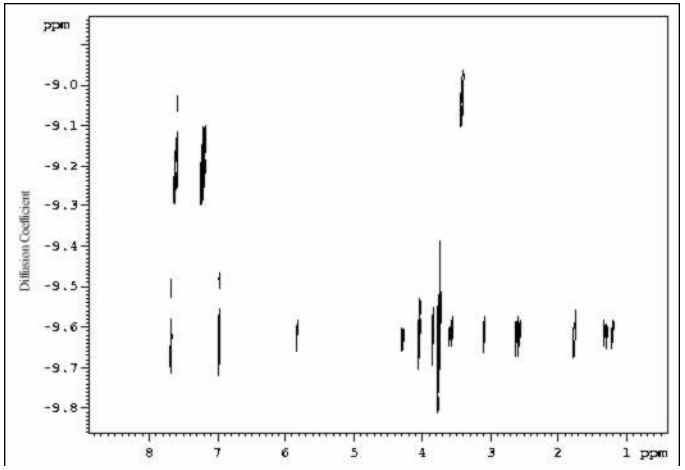
(Piccolo and Spiteller, manuscript)

Measurement of molecular masses by Diffusion NMR Spectroscopy

Liquid-state NMR spectroscopy allows a semi-quantitative evaluation of the molecular sizes of polydiserse substances by DOSY diffusion spectra which correlate molecular size with molecular motions under ^{1}H-NMR conditions.



DOSY Pulse sequence: ***stegs1s***



Diffusion is closely related to molecular size through the Stokes-Einstein equation

$$D=kT/6\pi\eta r$$

r=Hydrodynamic radius

Measurement of molecular masses by Diffusion NMR Spectroscopy

DOSY-NMR spectroscopy was applied to evaluate the molecular sizes of humic substances on the basis of calibration curves made with molecules of known MW.

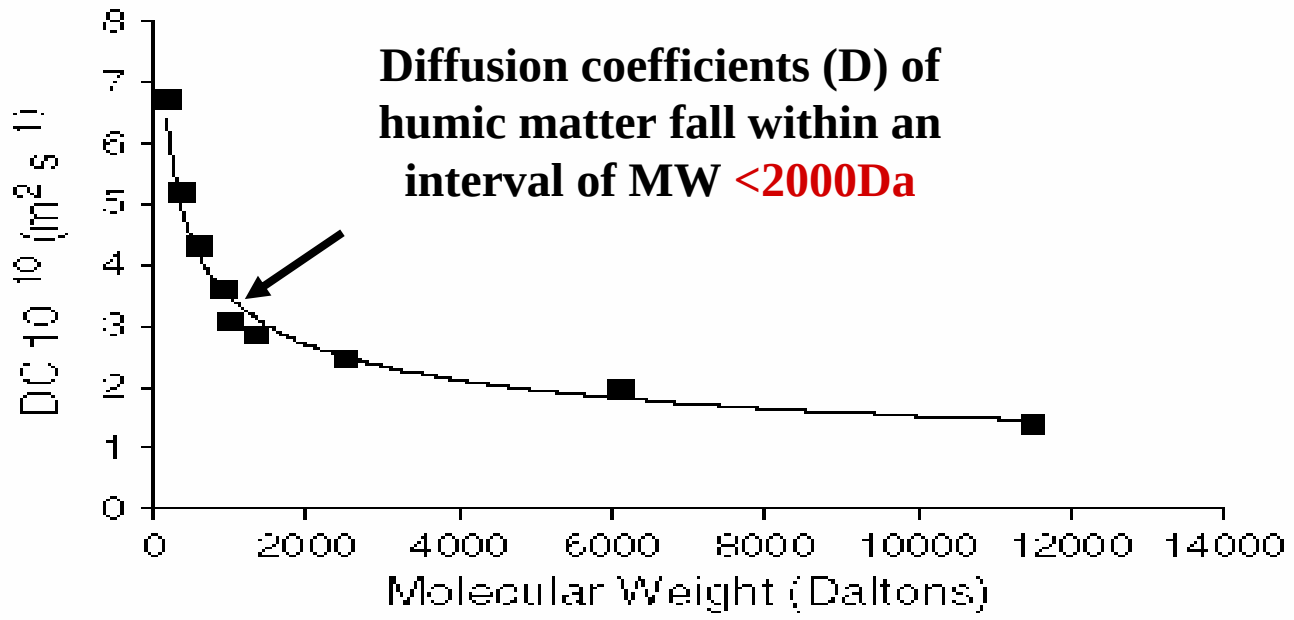


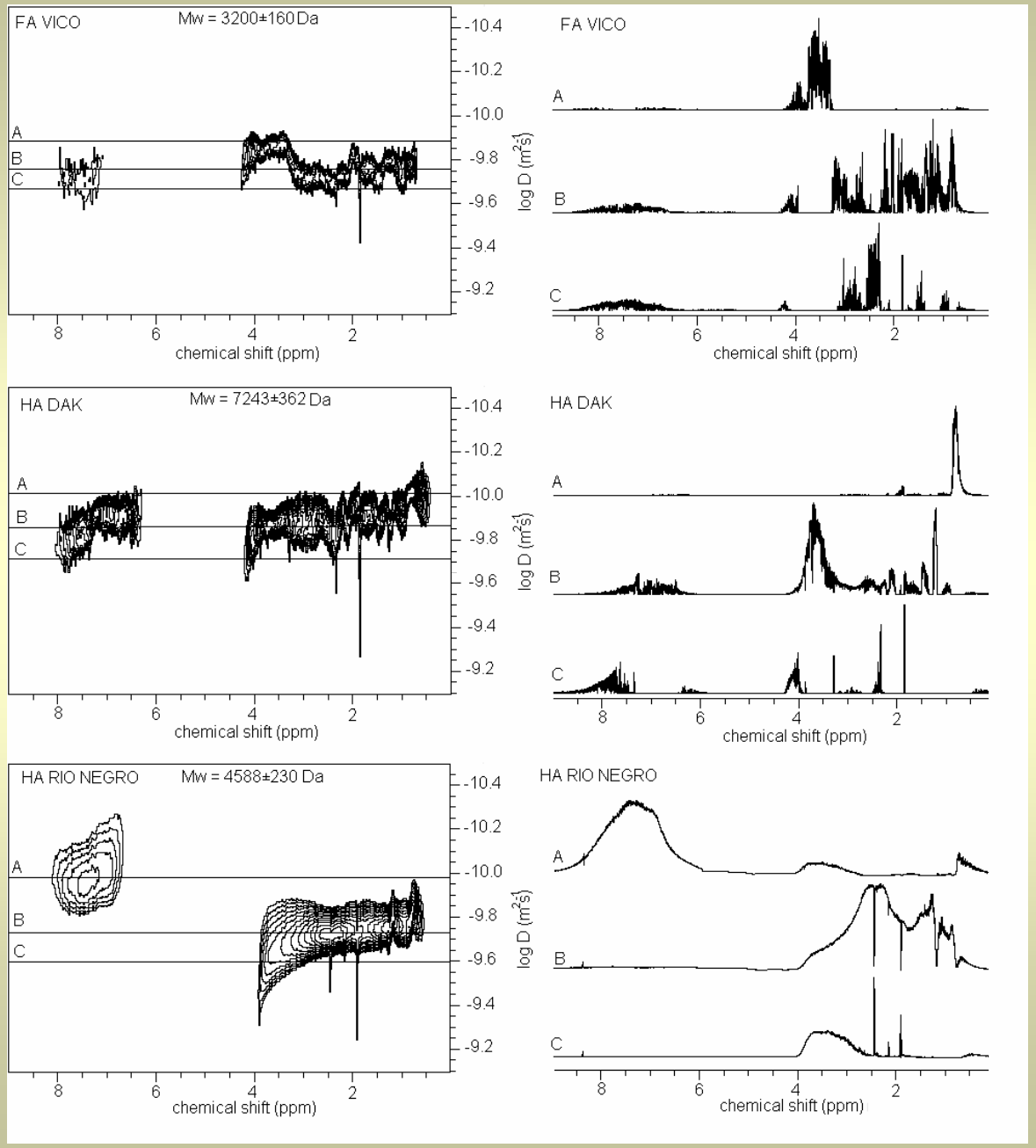
Figure 2. Diffusion coefficients (DC) of standard compounds and FSFA and PHA at 5 mg ml^{-1}. Standard compounds from left to right are glucose, sucrose, raffinose, γ-cyclodextrin, maltodextrin (910 Da), maltodextrin (1300 Da), insulin oxidized chain A, maltodextrin (6100 Da), insulin dimer.

Simpson. *Magnetic Resonance Chemistry*, 2002, 40: S72–S82

DOSY-NMR of humic and fulvic acids

Diffusion Order NMR Spectra evaluate the molecular size distributions of humic associations through measurements of diffusion coefficients (D).

The relative molecular sizes of different compound classes present in humic matter may be evaluated by extrapolating the 1D spectra associated to the pseudo 2D DOSY-NMR spectra

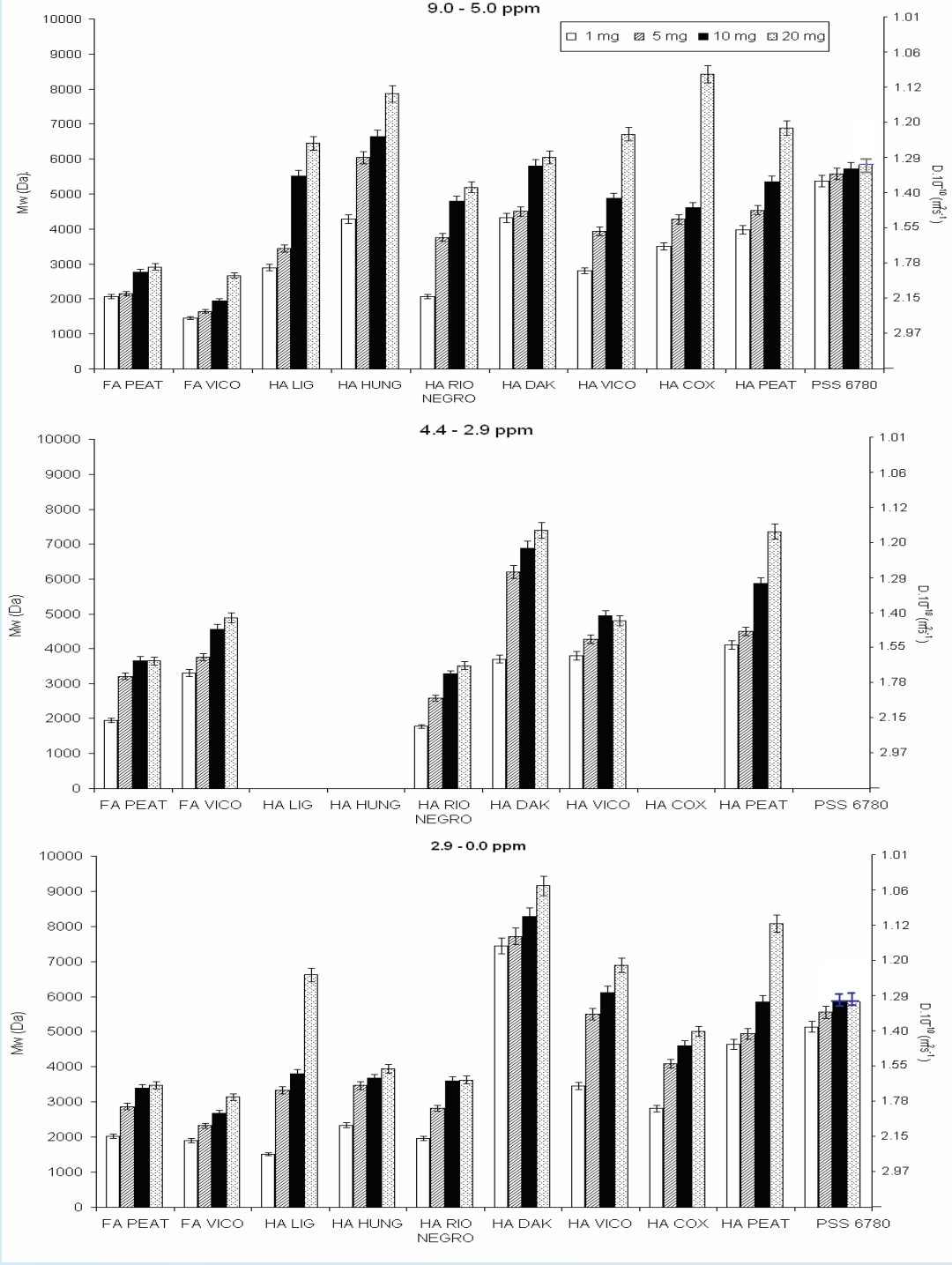


Smejkalova and Piccolo (2007). *Environmental Science and Technology*, submitted.

Aggregation

Changes of aggregation in 9 humic samples (pH 12.8) and one PSS standard (6780 Da), as a function of concentration, as revealed by DOSY-NMR Diffusion Coefficients (D) in different spectral regions

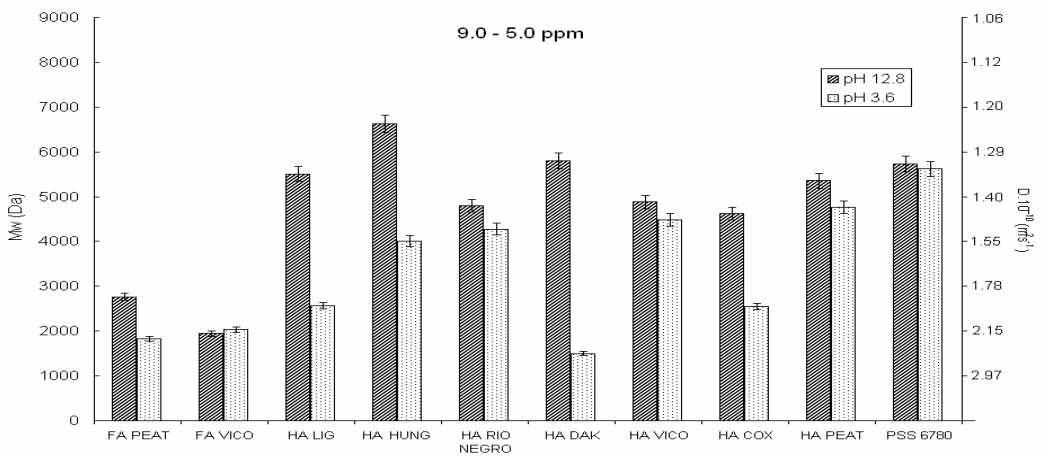
Increase in concentration produces a decrease of D as a result of increase in molecular size due to self-association of humic molecules

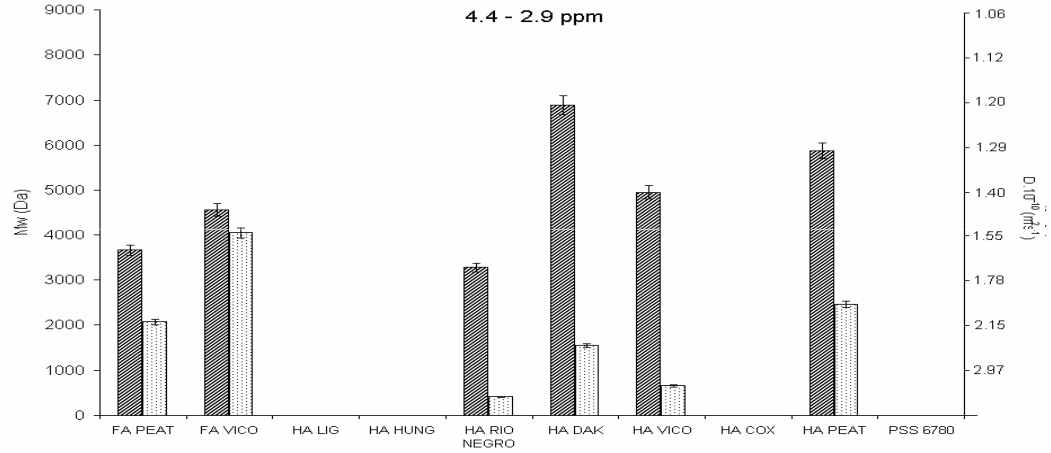


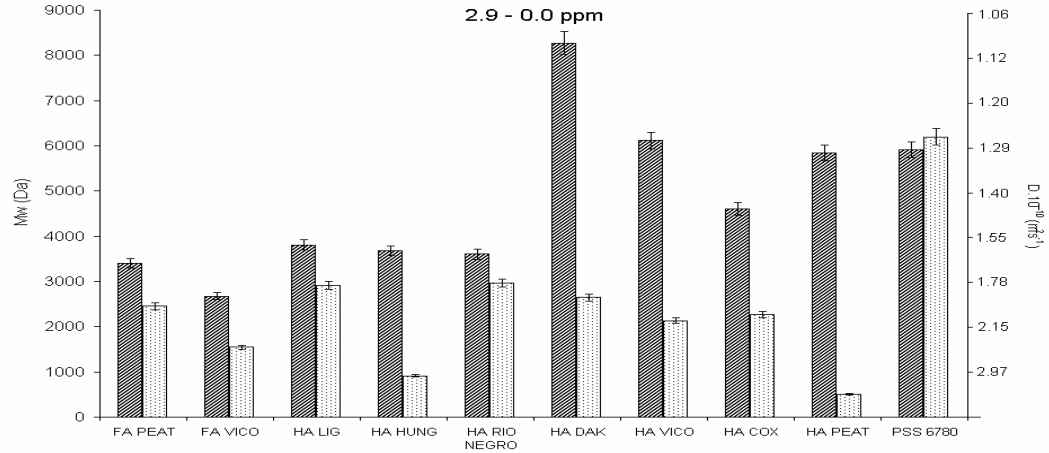
Disaggregation

Disaggregation in 9 humic samples and one PSS standard (6780 Da) when acetic acid was added to 10 mg.mL^{-1} solution down to pH 3.6 as revealed by DOSY-NMR Diffusion Coefficients (D) in different spectral regions.

The pH reduction of solutions produced dramatic decrease in molecular size following the disrupture of humic supramolecular structures







THE NEW HYPOTHESIS

Humic substances are composed of relatively small and heterogeneous molecules that self-assembled in supramolecular associations of only apparently large molecular sizes. The associations are stabilized by weak bonds (H-bonds, π-π, CH-π) and the hydrophobic effect that enhances solvation entropy in water.

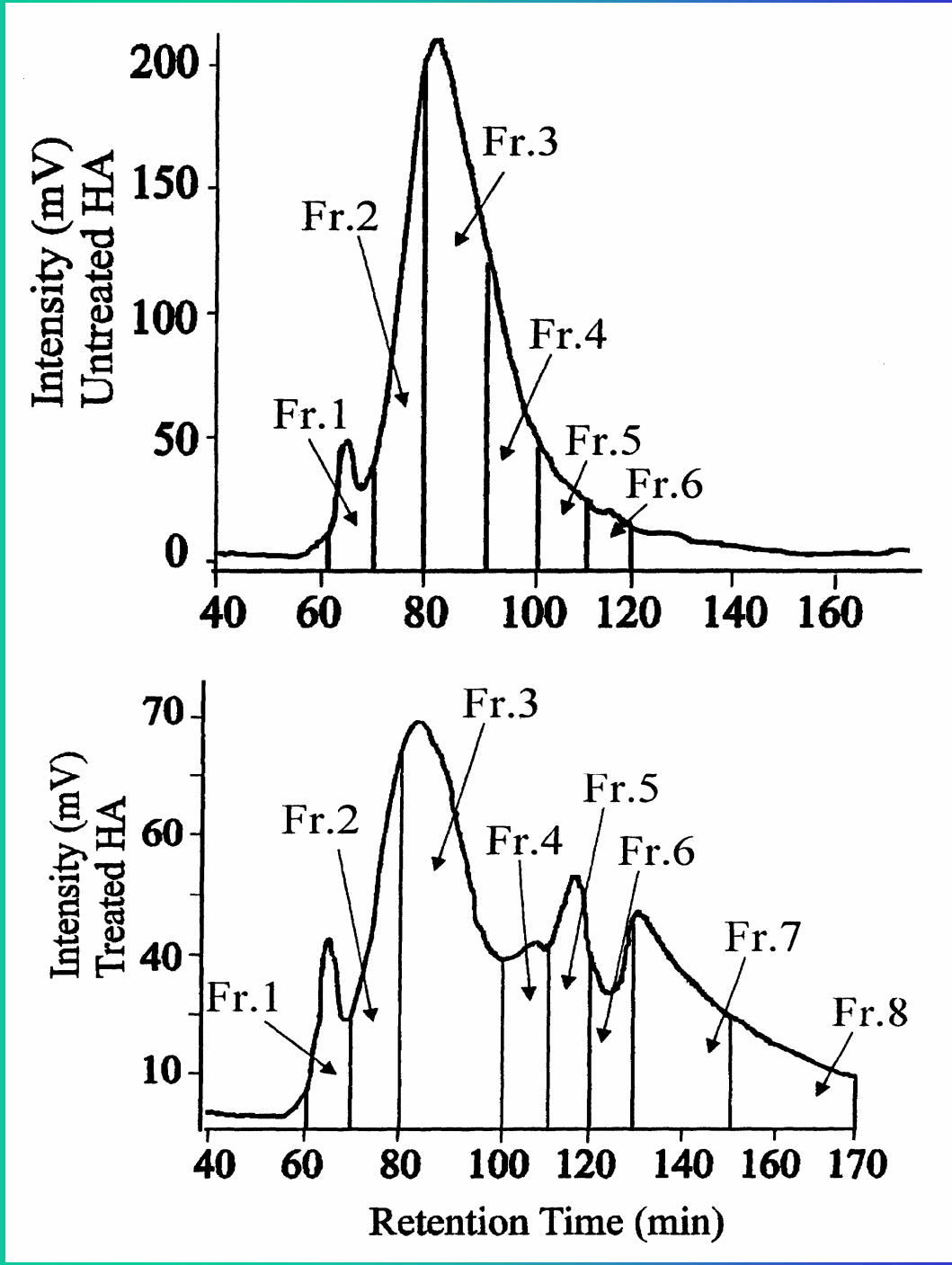
The humic molecules, are carbonaceous compounds surviving the biochemical degradation of various components, randomly associate in hydrophobic-hydrophilic phases which are either contiguous to or contained in each other. Their environmental reactivity is dictated by their hydophobic/hydrophilic ratio

The energetically labile supramolecular associations may be altered by interactions with metals and organic molecules that are stronger than hydrophobic bonds, and stabilize the original humic conformation. Then, the supramolecular structure changes conformational stability and energy content.

THE SUPRAMOLECULAR NATURE OF HUMIC SUBSTANCES CAN BE DETERMINED IN DIFFERENT WAYS:

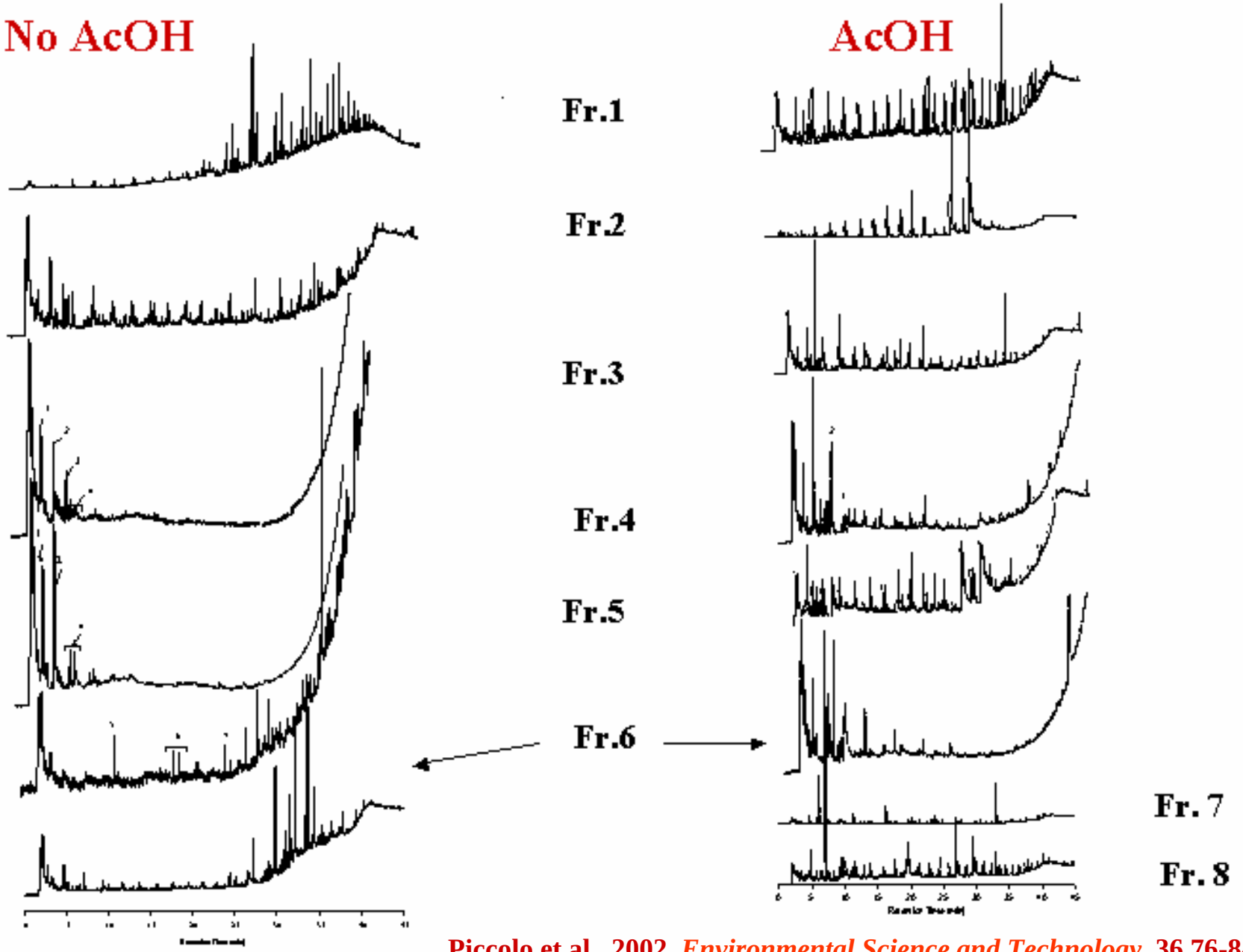
1. Separation in smaller-size humic associations by preparative HPSEC in order to reach a better insight of their molecular structure

Preparative HPSEC elution of a humic acid from lignite in NaCl 0.05 M, pH 7, before and after addition of acetic acid to pH 3.5



Piccolo et al., 2002. *Environmental Science and Technology*, 36,76-84

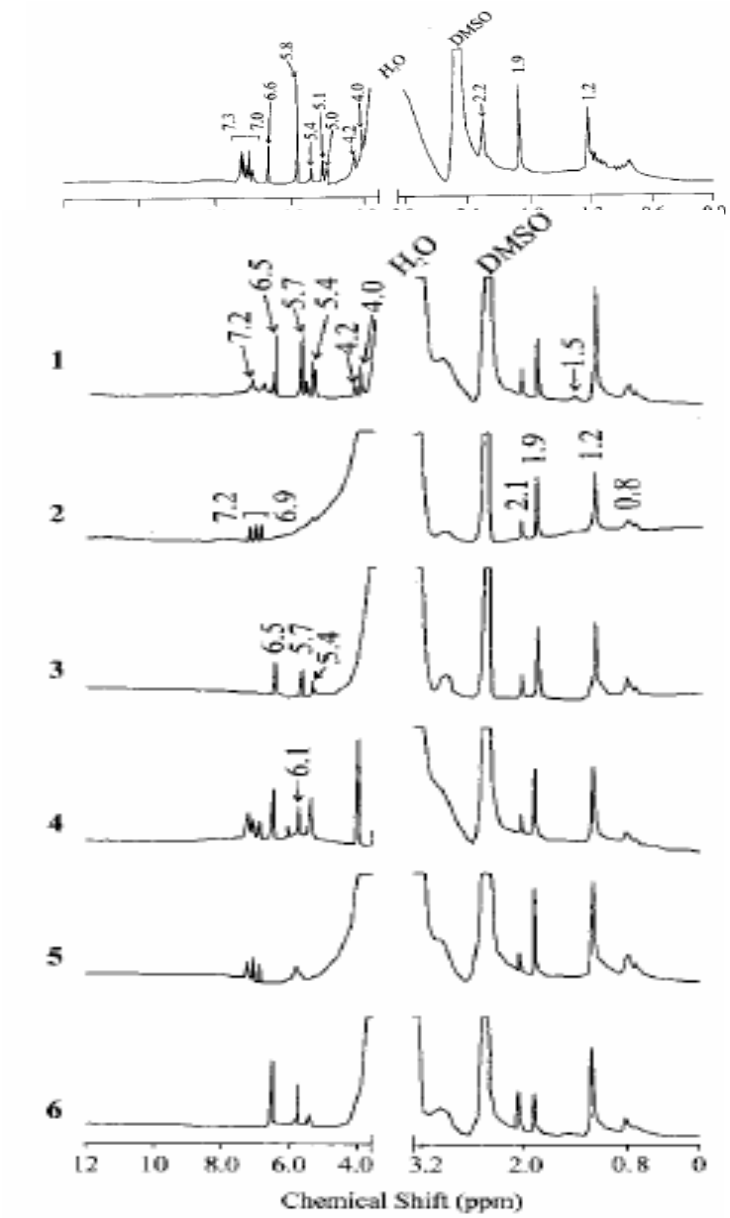
Pyrolysis (Curie-Point)-GC-Mass Spectrometry



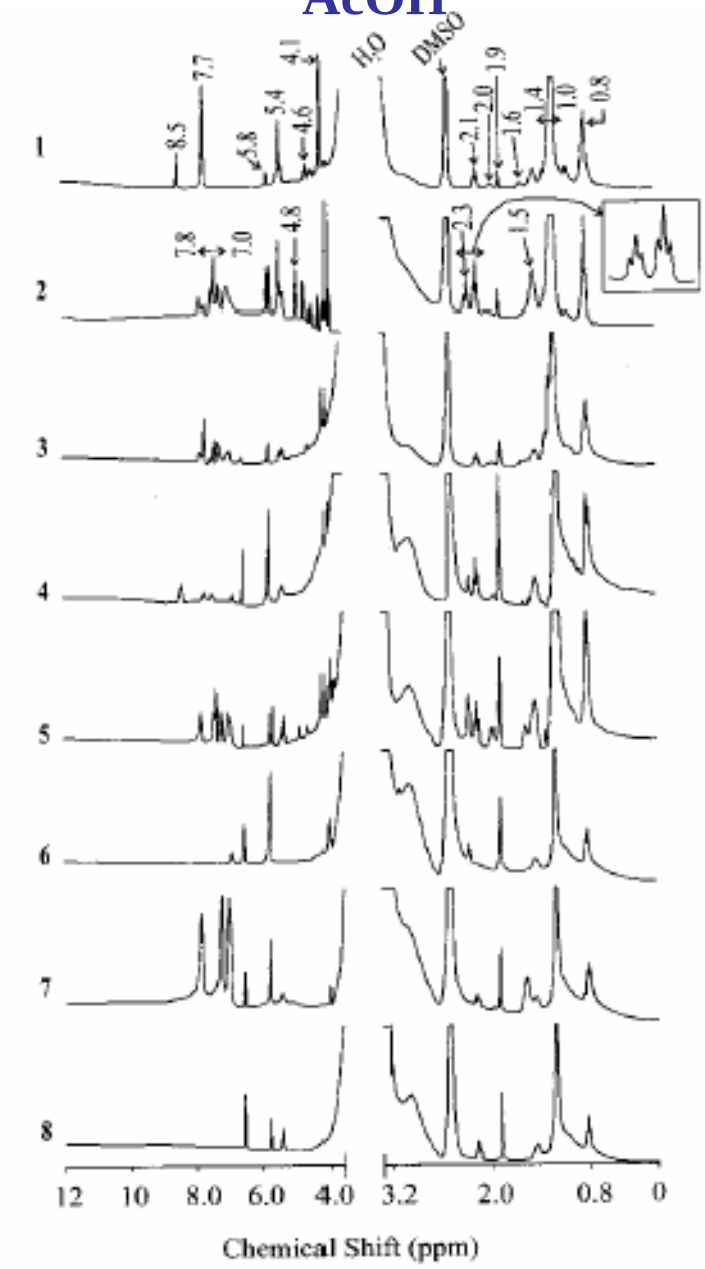
Piccolo et al., 2002. *Environmental Science and Technology*, 36,76-84

1D-^{1}H-NMR spectra of fractions separated by preparative HPSEC from a lignite HA, before and after AcOH treament

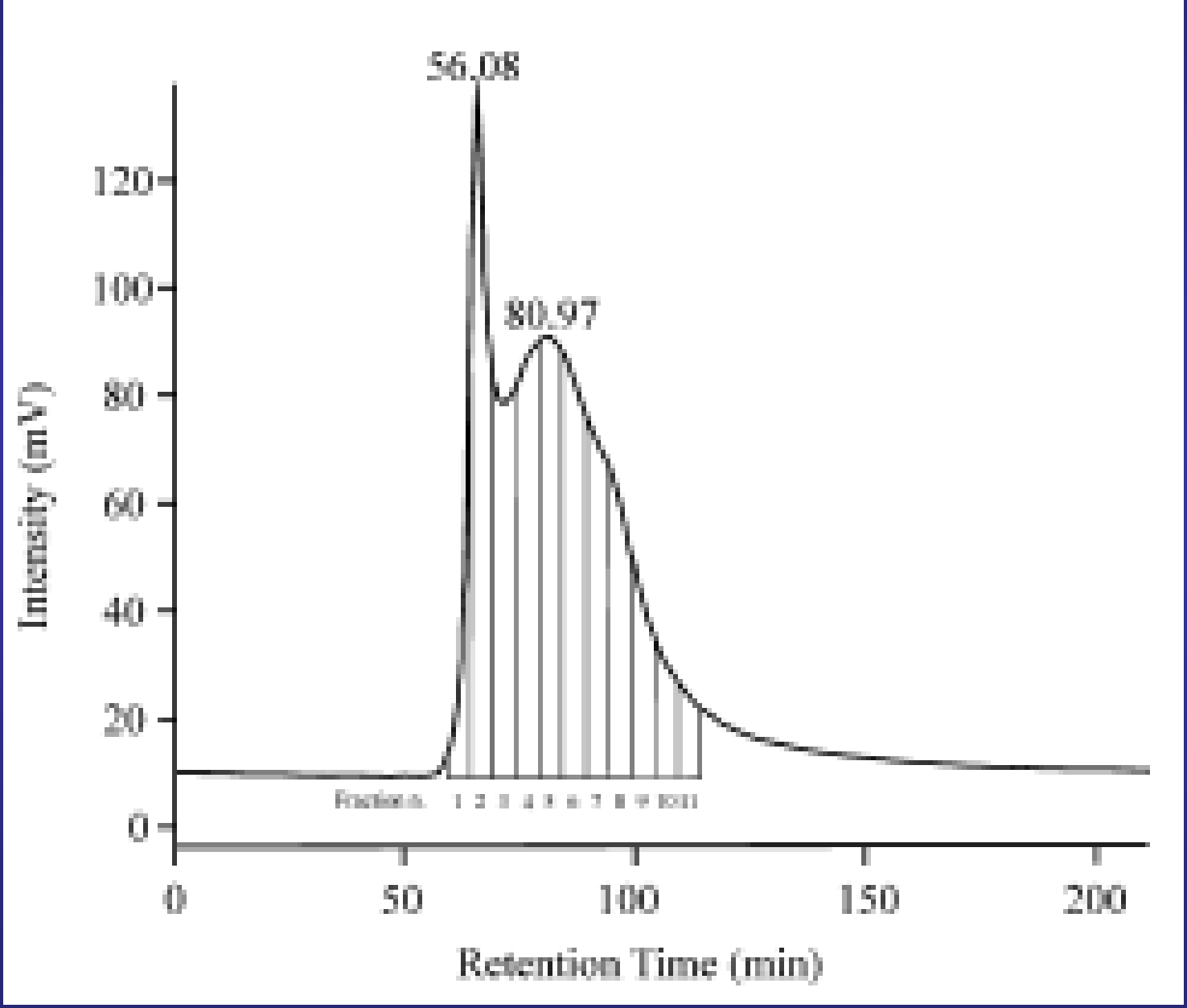
No AcOH



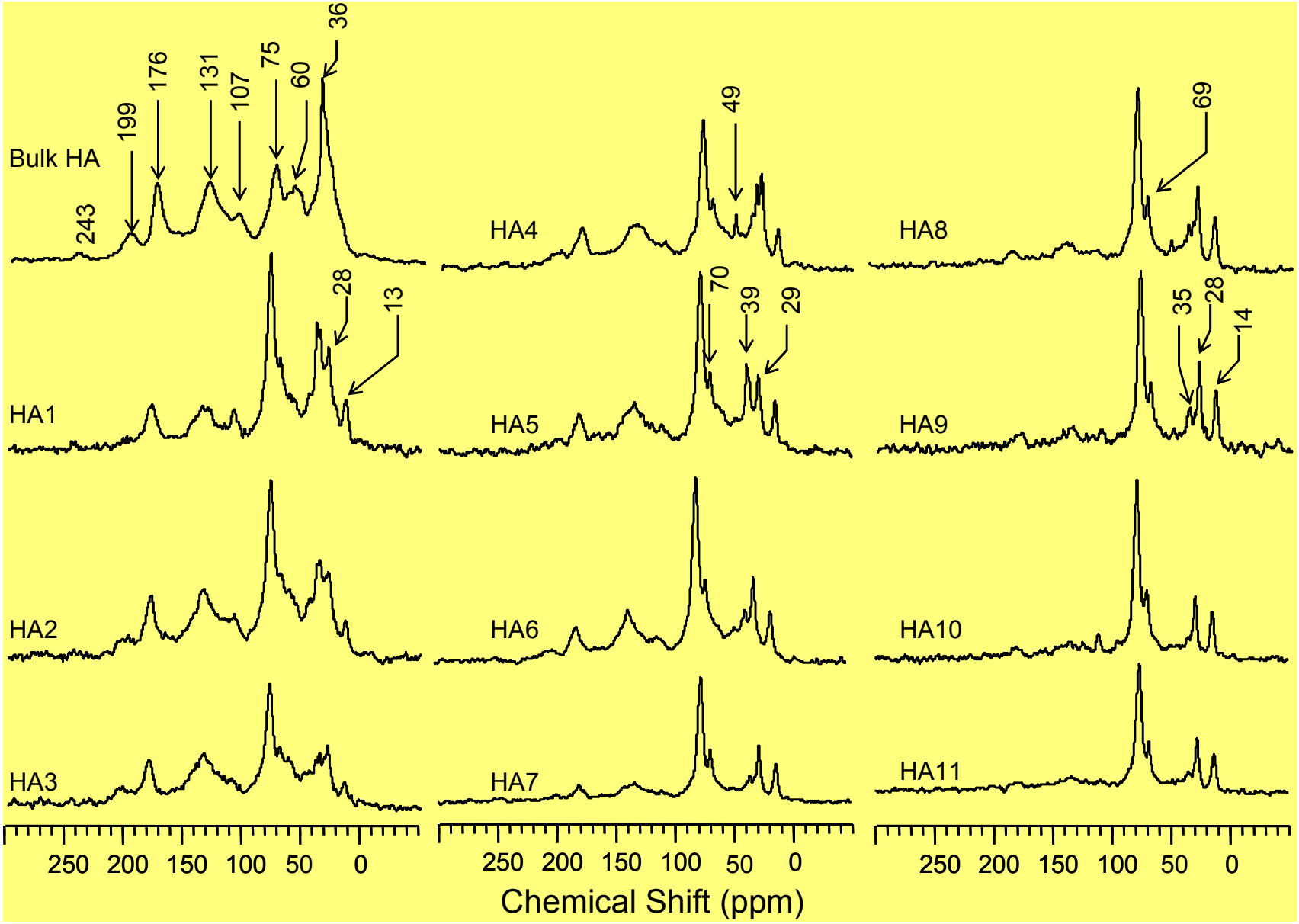
AcOH



Preparative HPSEC isolation of 11 size-fraction separated from HA of a volcanic soil



^{13}C-CPMAS-NMR spectra of fractions separated by preparative HPSEC from HA of a volcanic soil



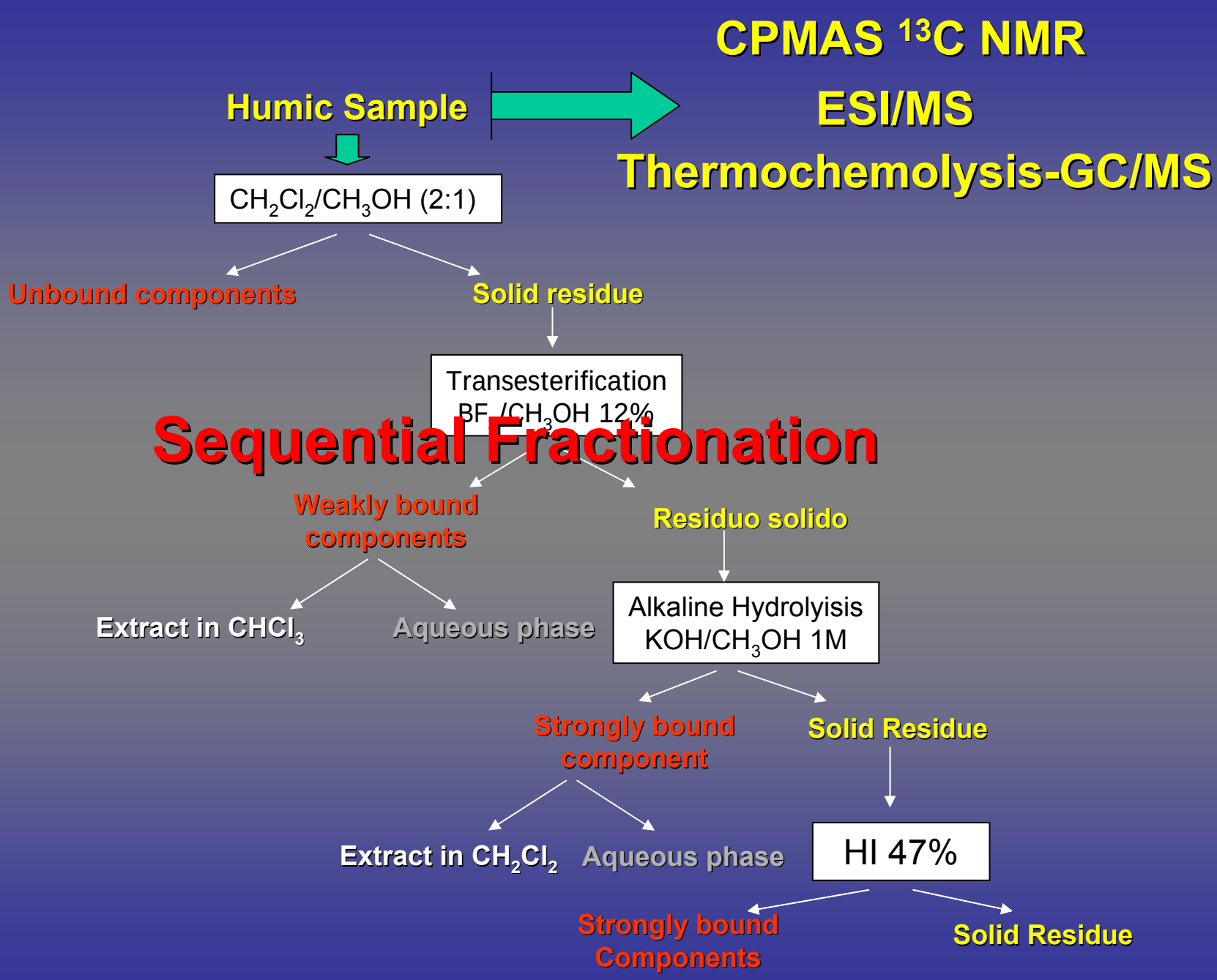
THE SUPRAMOLECULAR NATURE OF HUMIC SUBSTANCES CAN BE DETERMINED IN DIFFERENT WAYS:

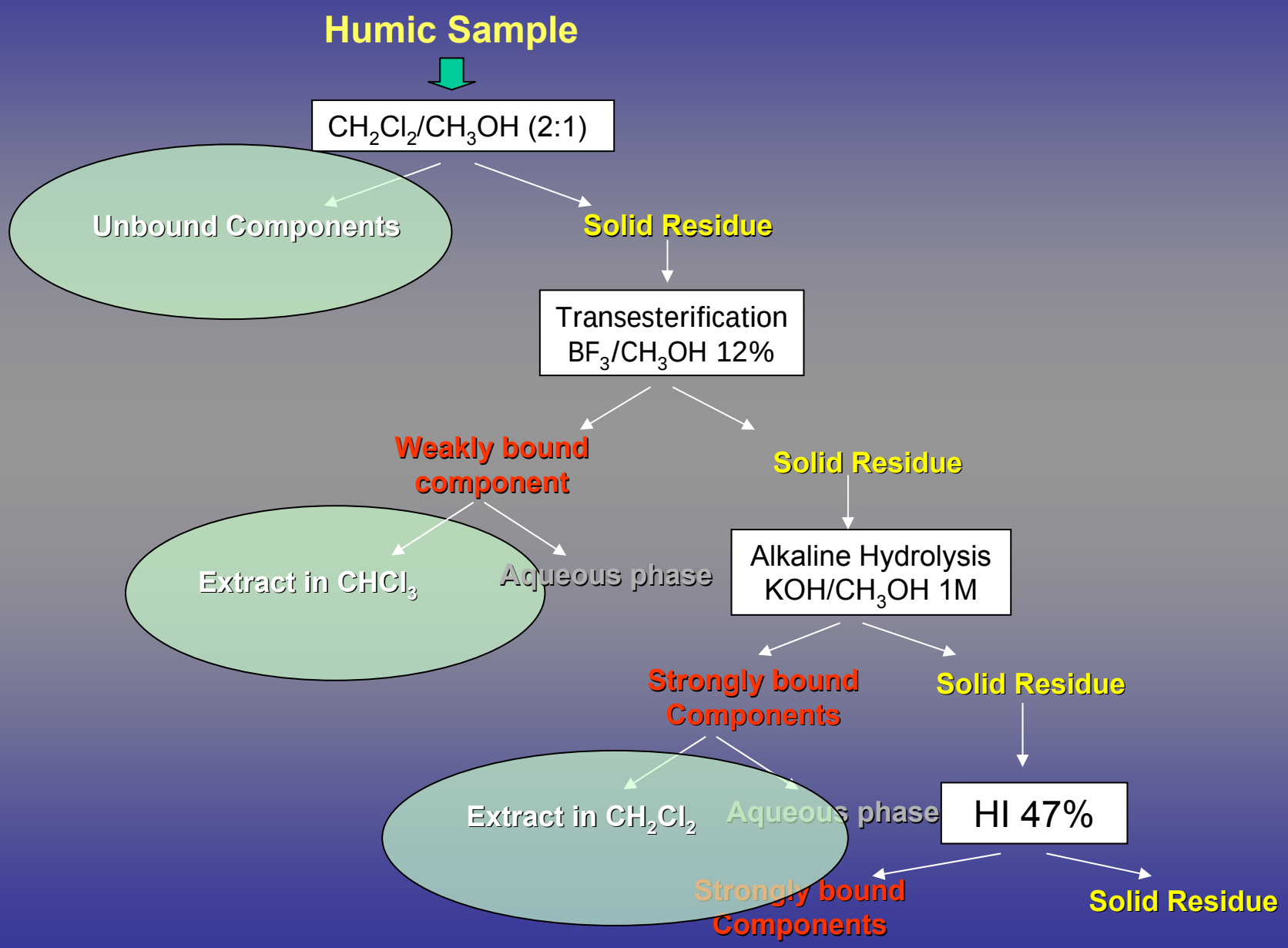
2. The heterogeneous components of humic substances may be selectively separated and analytically determined one by one in order to reach a better understanding of the detailed molecular structure.

Sequential Chemical Fractionation
by progressively removing molecular classes

1. Molecules structurally **unbound** to the humic matrix by organic solvent extraction
2. Molecules **weakly bound** to the humic matrix through **ester bonds** by transesterification (BF_3-MeOH)
3. Molecules **strongly bound** to the humic matrix through **ester bonds** by alkaline hydrolysis in MeOH
4. Molecules **strongly bound** to the humic matrix through **ether bonds** by HI treatment

Sequential Fractionation




Humic Sample
CH_2Cl_2/CH_3OH (2:1)
Unbound Components
Solid Residue
Transesterification
BF_3/CH_3OH 12%
Weakly bound component
Solid Residue
Extract in $CHCl_3$
Aqueous phase
Alkaline Hydrolysis
KOH/CH_3OH 1M
Strongly bound Components
Solid Residue
Extract in CH_2Cl_2
Aqueous phase
HI 47%
Strongly bound Components
Solid Residue

Unbound components

SPE

$CH_2Cl_2/CH_3CH(OH)CH_3$ (2:1)
Neutral Fraction

$CH_3CH_2OCH_2CH_3/CH_3COOH$ (98:2)
Acid Fraction

Extract in $CHCl_3$

SPE

$CH_2Cl_2/CH_3CH(OH)CH_3$ (2:1)
Neutral Fraction

$CH_3CH_2OCH_2CH_3/CH_3COOH$ (98:2)
Acid Fraction

Extract in CH_2Cl_2

SPE

$CH_2Cl_2/CH_3CH(OH)CH_3$ (2:1)
Neutral Fraction

$CH_3CH_2OCH_2CH_3/CH_3COOH$ (98:2)
Acid Fraction

Gas-chromatography/mass-spectrometry

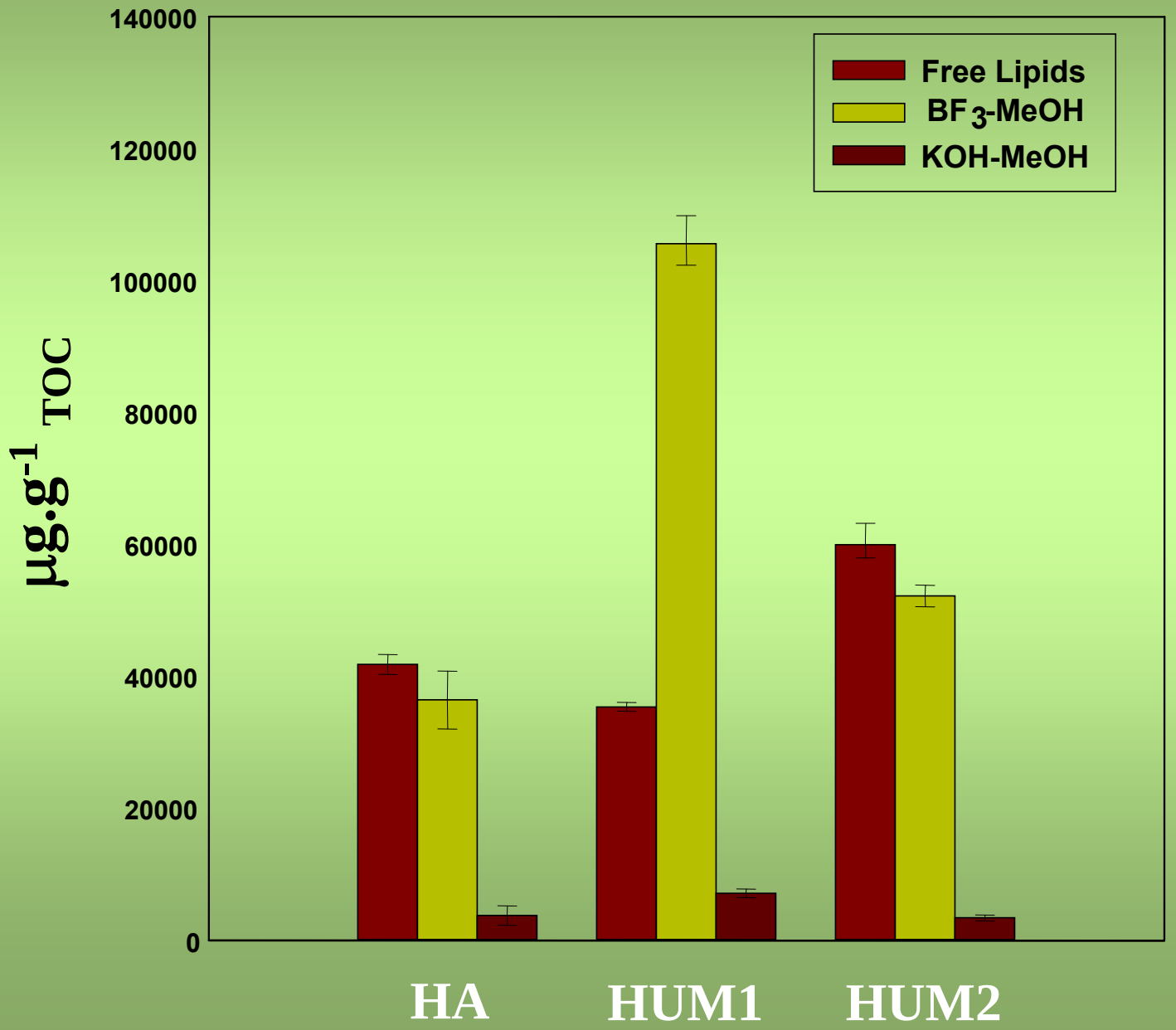
The proposed sequential fractionation was applied to the following samples:

1. **HA** **Humic acid extracted from a volcanic soil**
2. **HUM1** **Humin remaining in soil after HA extraction**
3. **HUM2** **Humin remaining in soil after treatment of HUM1 with a 10% HF solution**

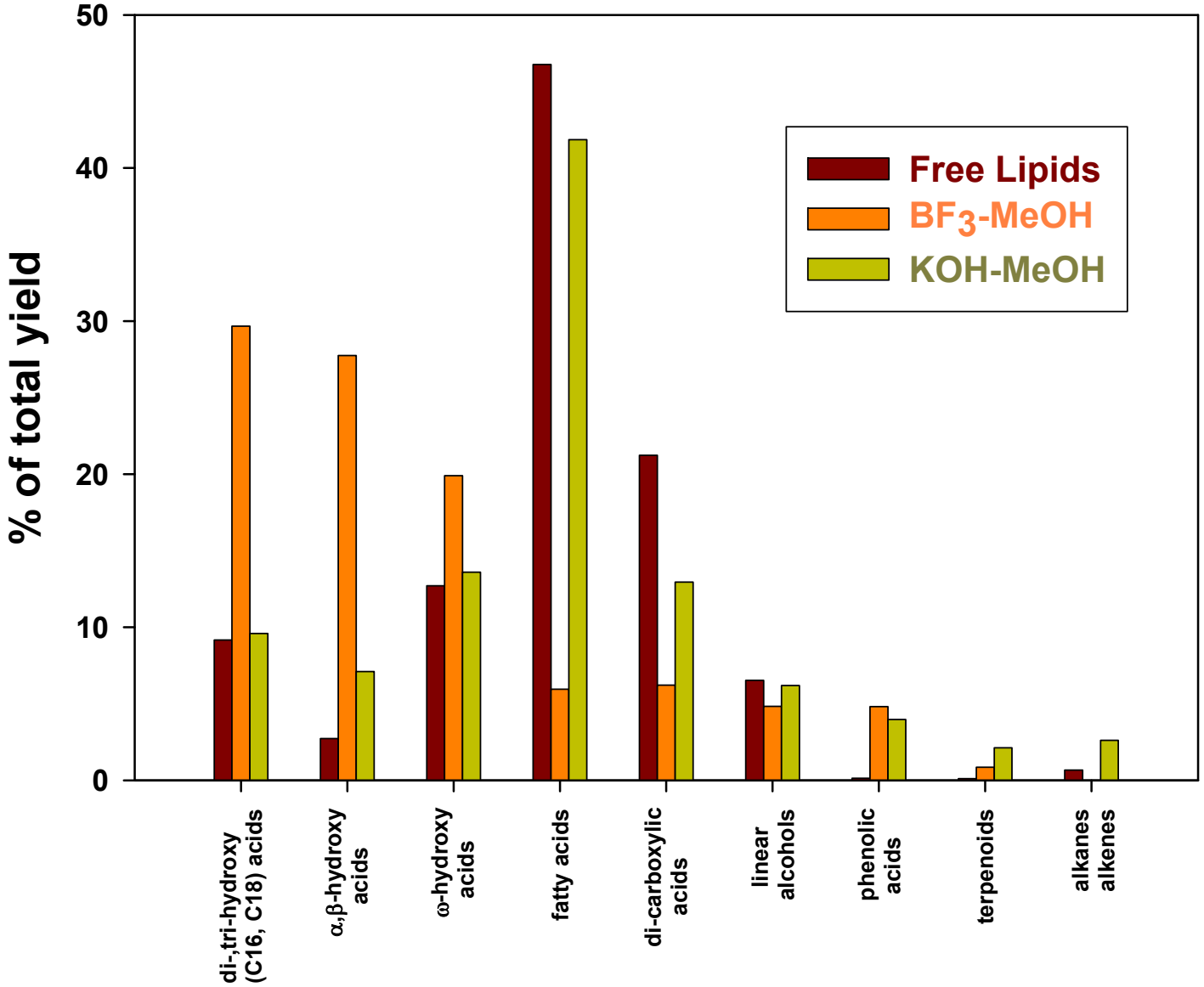
and

1. **HAT** **Titrated and quartz-filtered HA**
2. **F1** **HPSEC separated high MW fraction of HAT**
3. **F2** **HPSEC separated medium MW fraction of HAT**
4. **F3** **HPSEC separated low MW fraction of HAT**

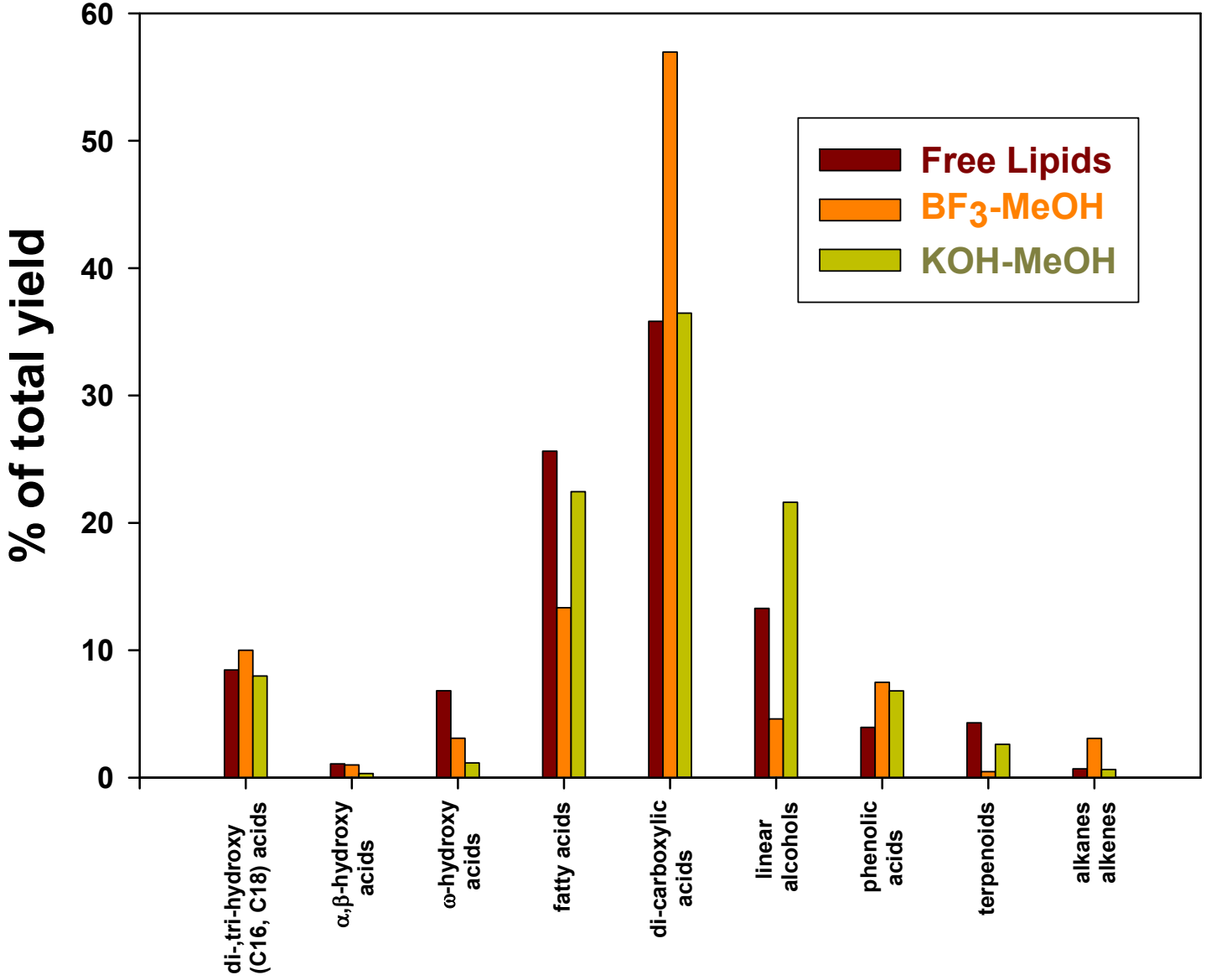
Total yields of compounds by GC-MS



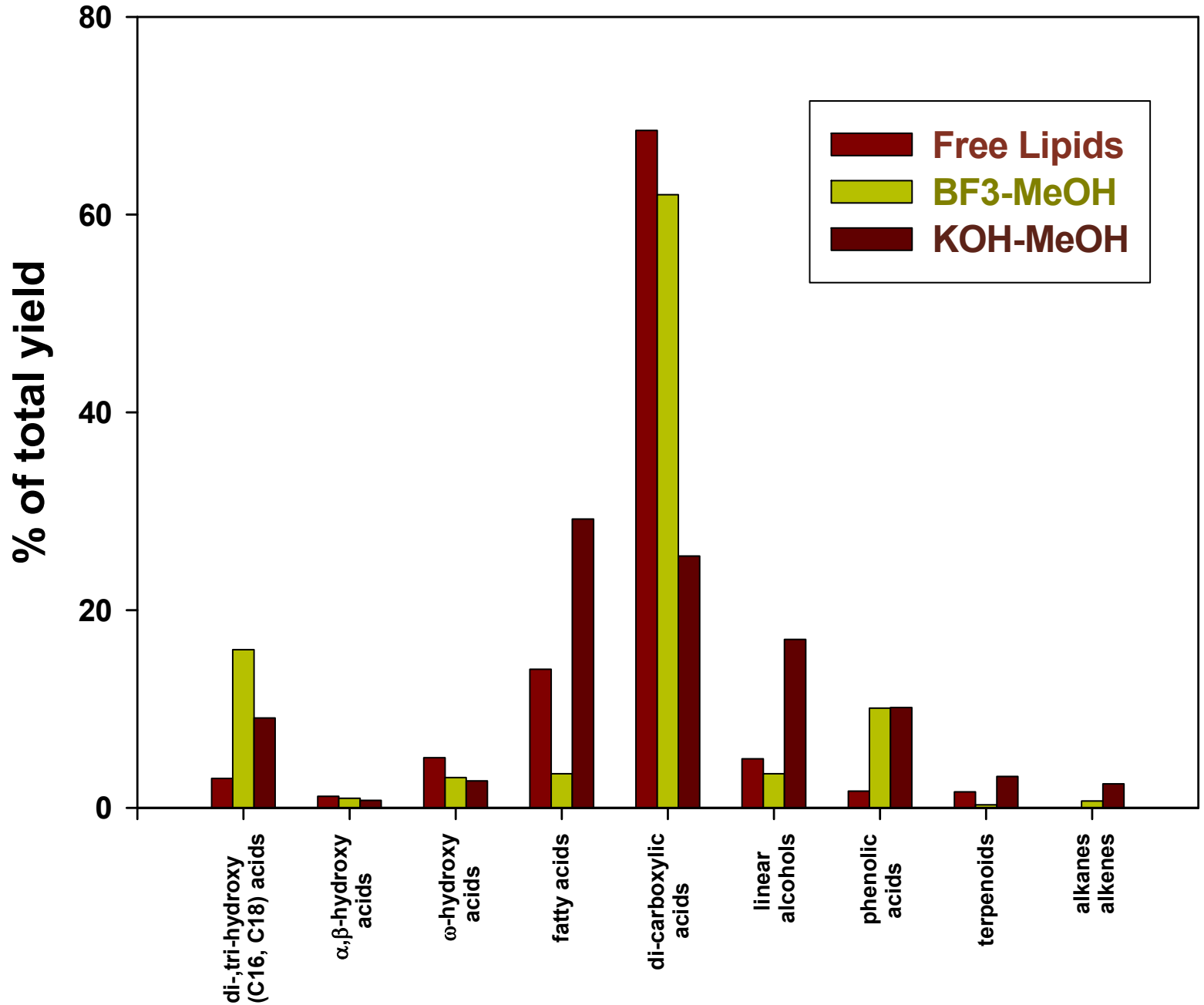
Classes of compounds found in HA as % of total yield



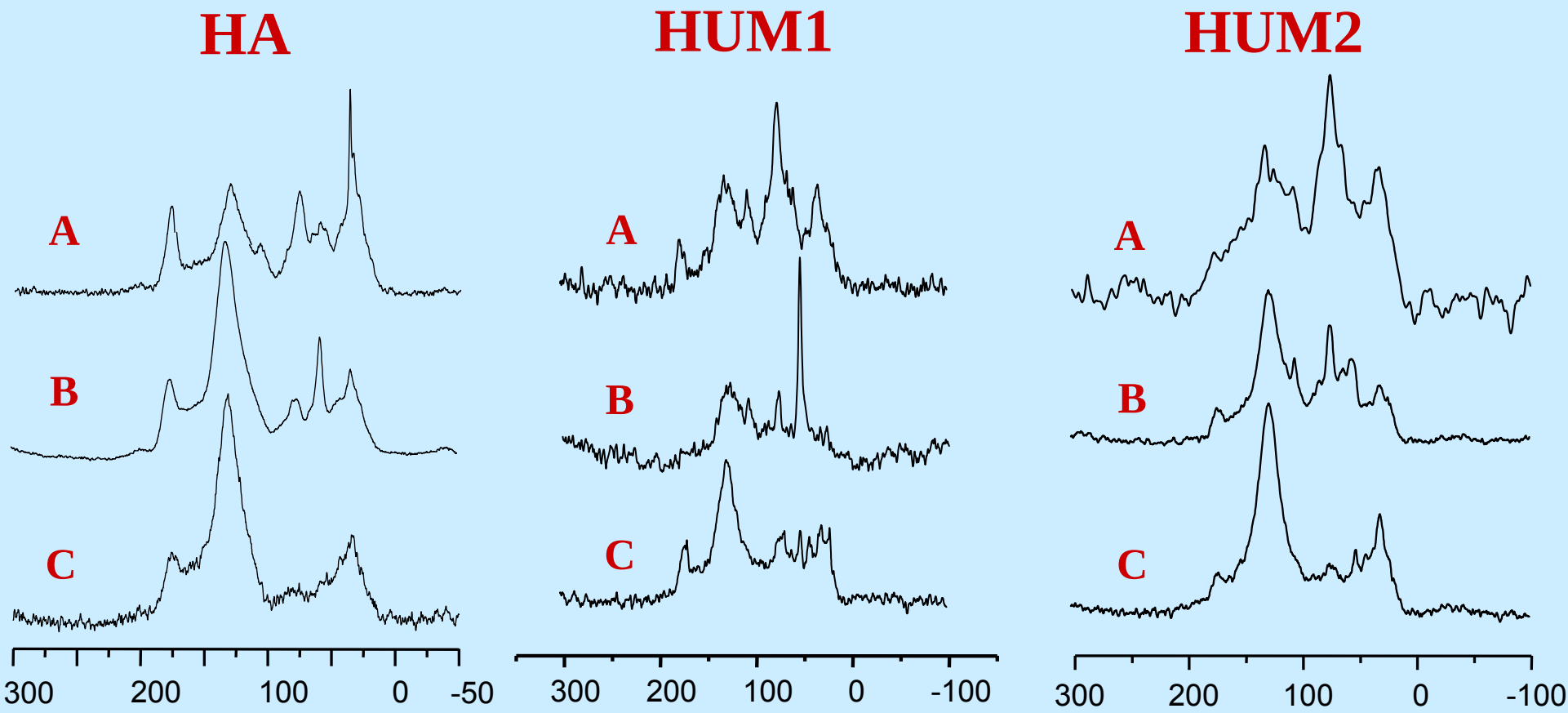
Classes of compounds found in HUM1 as % of total yield



Classes of compounds found in HUM2 as % of total yield



CPMAS-^{13}C-NMR spectra of humic samples



A. INITIAL BULK

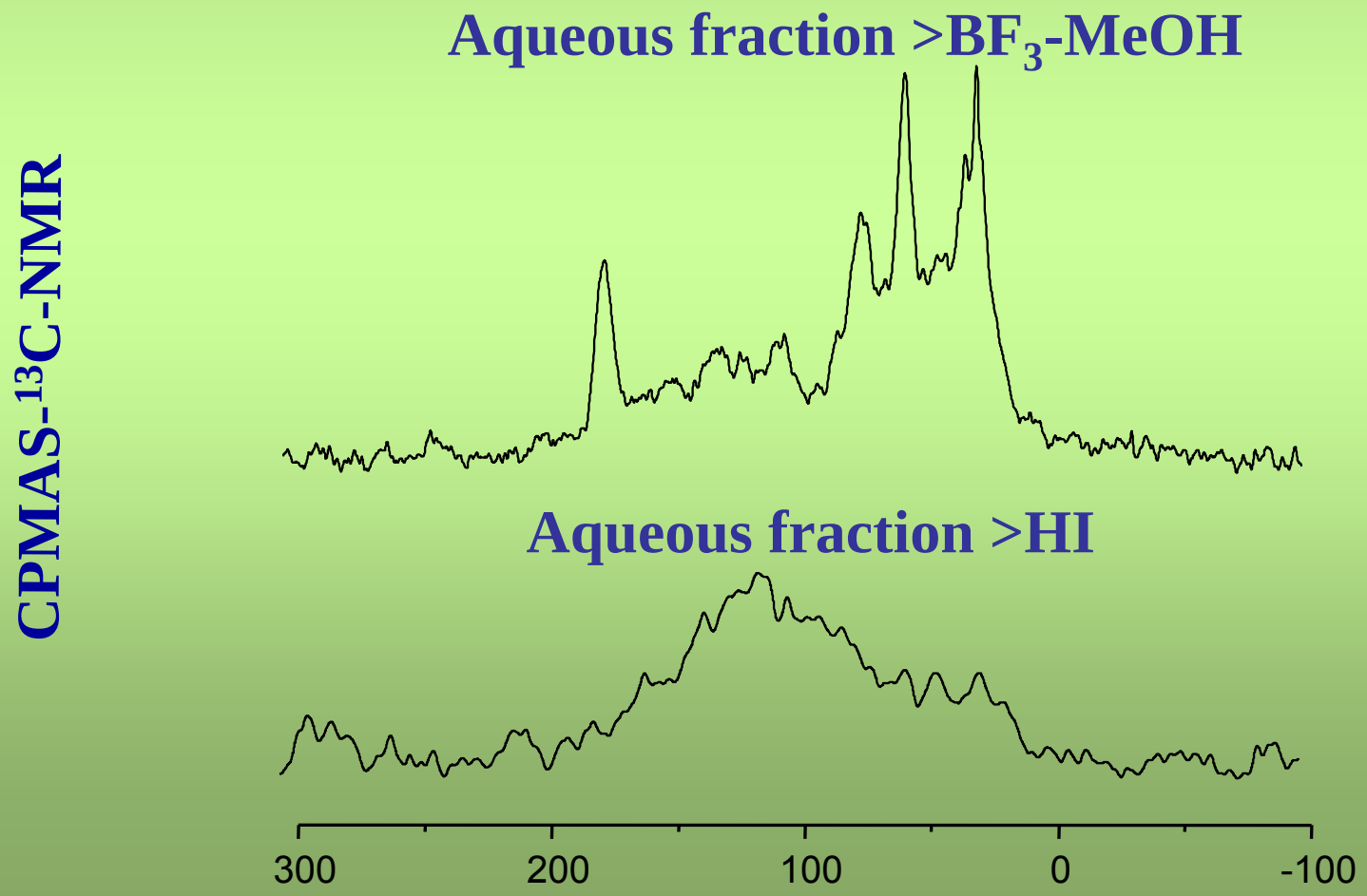
B. RESIDUE >KOH-MeOH

C. RESIDUE >HI

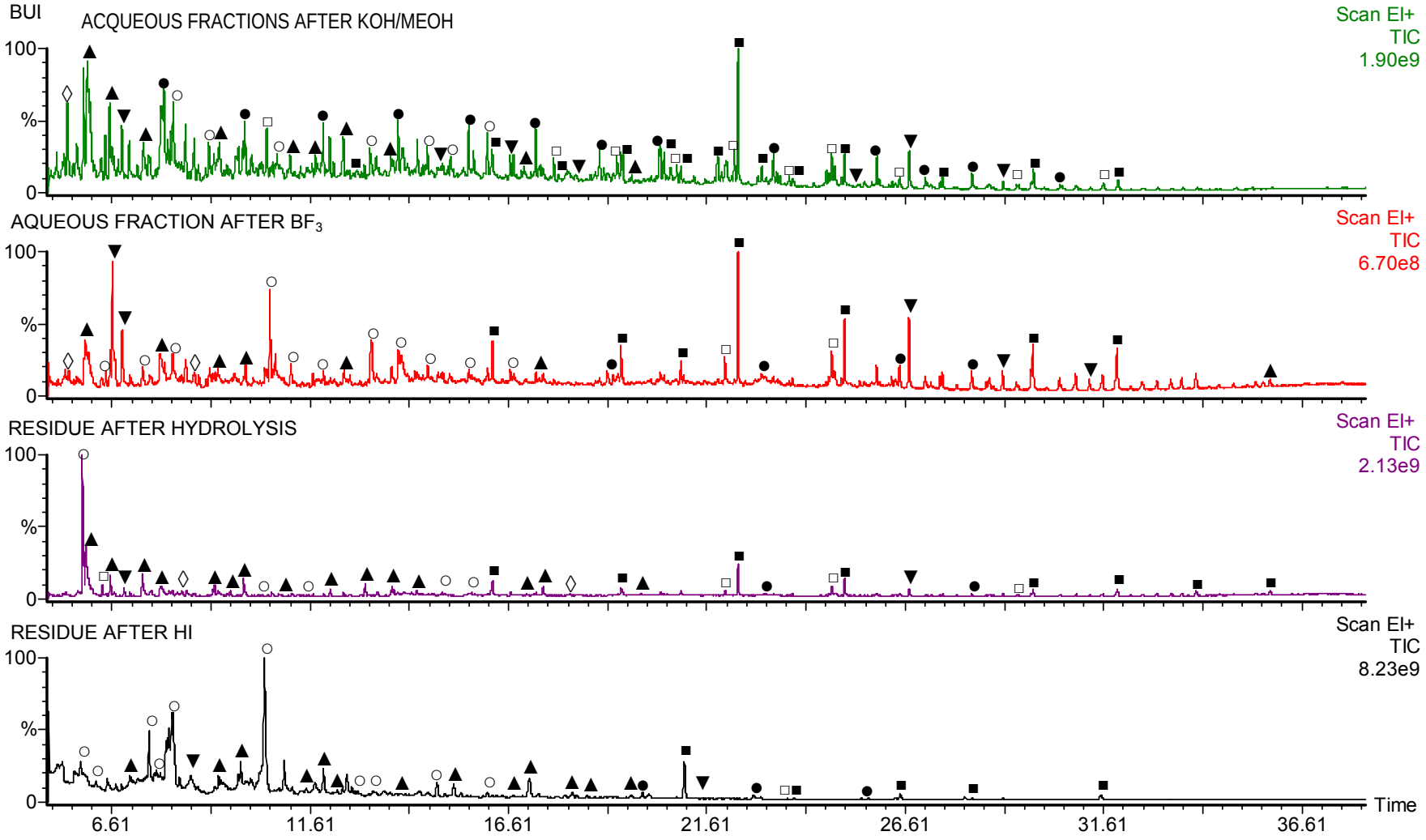
Percent of extraction yields (w/w)

HA		HUM1		HUM2	
>KOH MeOH	>HI	>KOH MeOH	>HI	>KOH MeOH	>HI
60-70	80-87	30-40	80-83	45-50	56-60

The aqueous fractions resulting from the sequential fractionation cannot be analyzed by GC-MS. However, their chracterization may be achieved by NMR, Thermochemolysis, and ESI-MS



On-Line thermochemolysis (TMAH) of humic samples from fractionation of HA



■ satured monocarboxilic acid □ unsatured monocarboxilic acid

● alkene/alkane dublet ○ nitrogen containing compound

▼ dicarboxylic acid ▲ phenolic compound

◊ polysaccharide derived compound

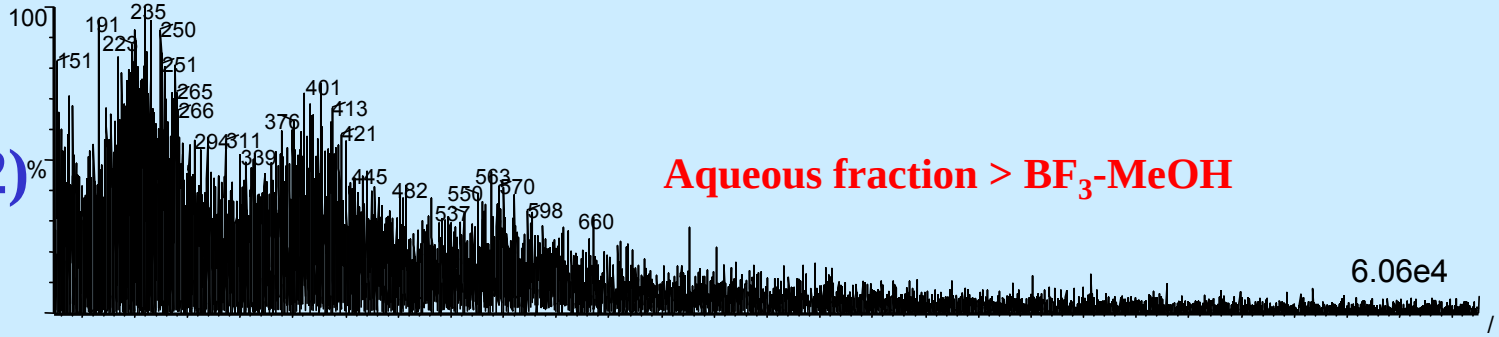
ESI-MS spectra of humic fractions of HA

Mw

909(±30)

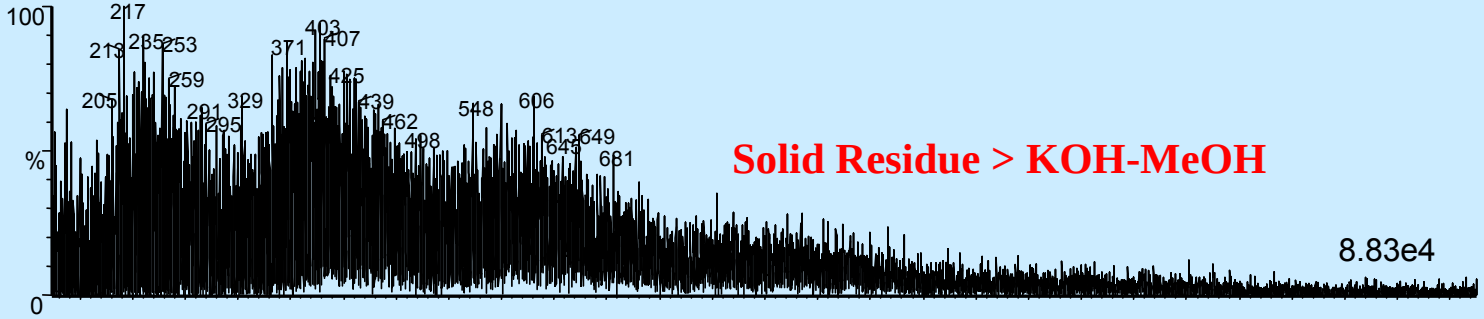
Bulk HA

735(±22)



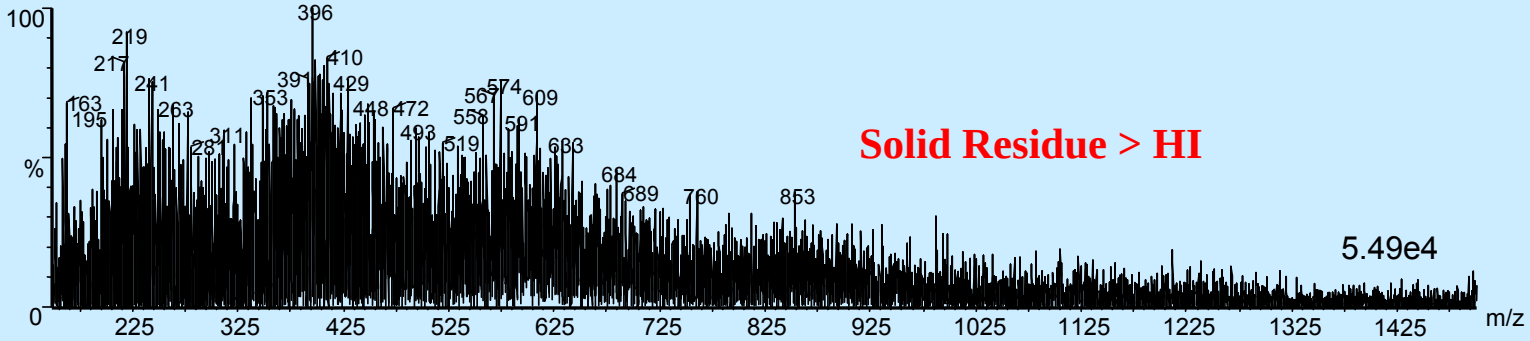
Aqueous fraction > BF_3-MeOH

727(±8)



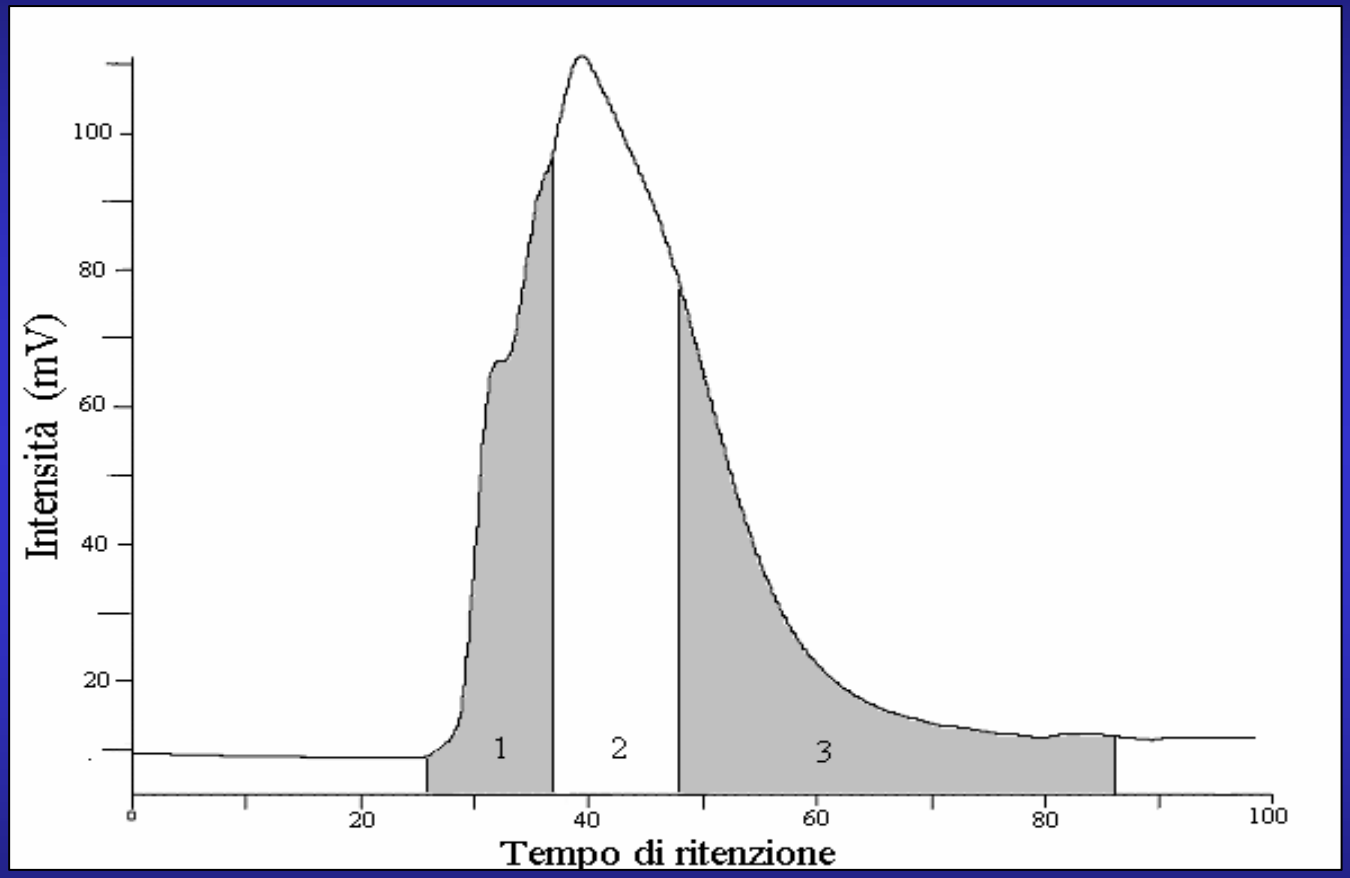
Solid Residue > KOH-MeOH

782(±1)



Solid Residue > HI

SIZE-FRACTIONS SEPARATION FROM HA BY PREPARATIVE HPSEC



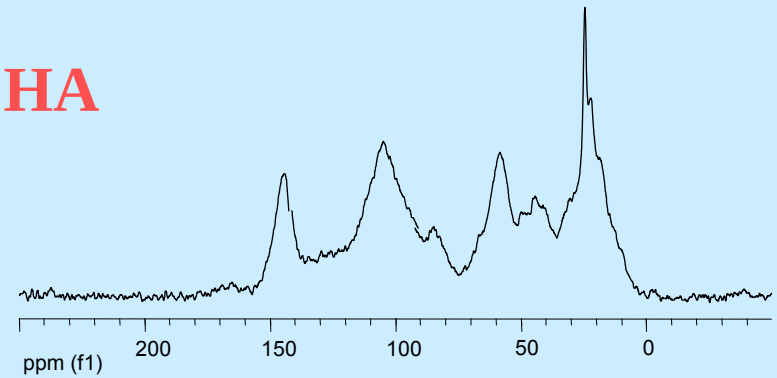
Fraction 1
High Molecular Sizes

Fraction 2
Intermediate Molecular Sizes

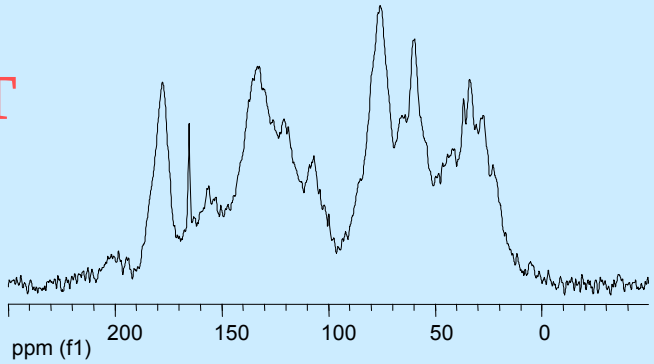
Fraction 3
Low Molecular Sizes

CPMAS-^{13}C-NMR spectra of humic samples

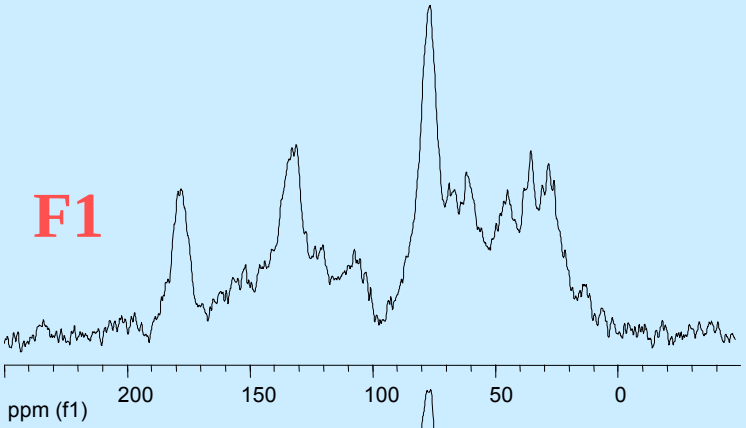
HA



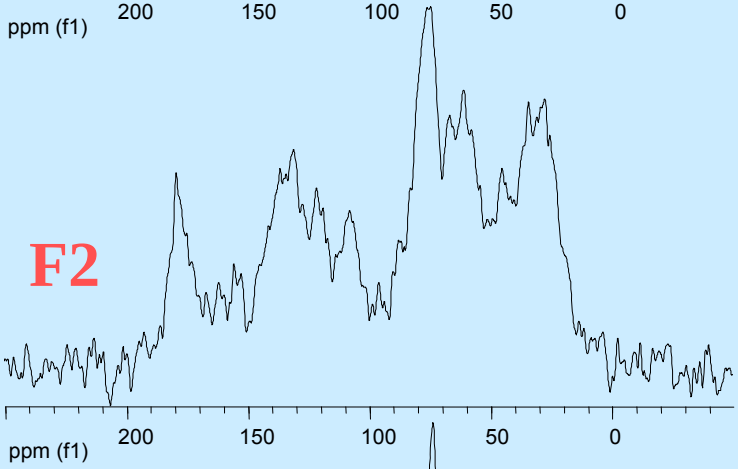
HAT



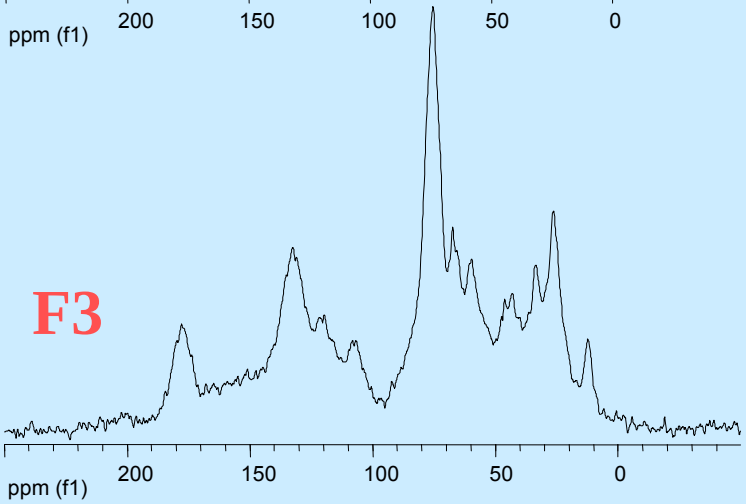
F1



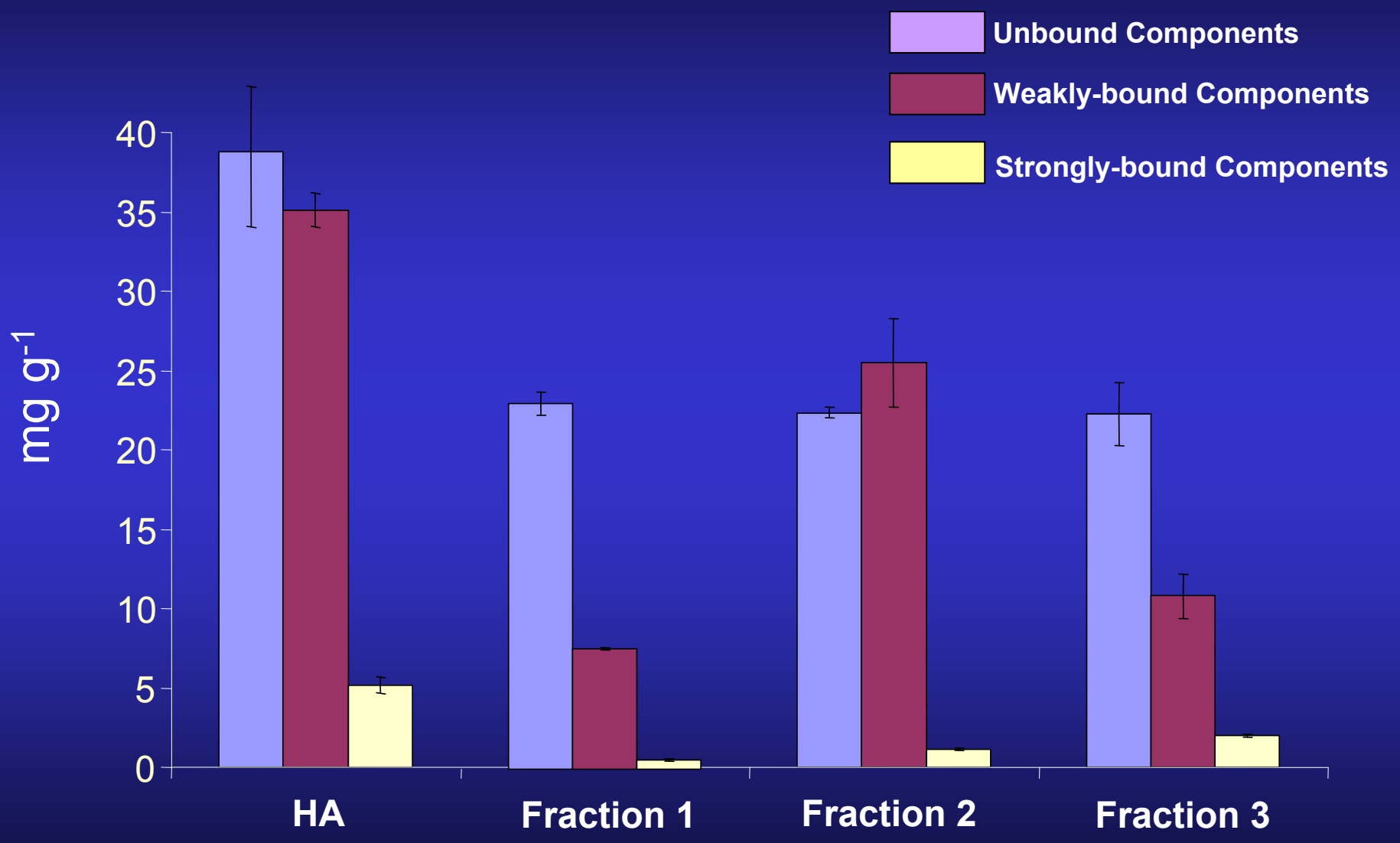
F2



F3

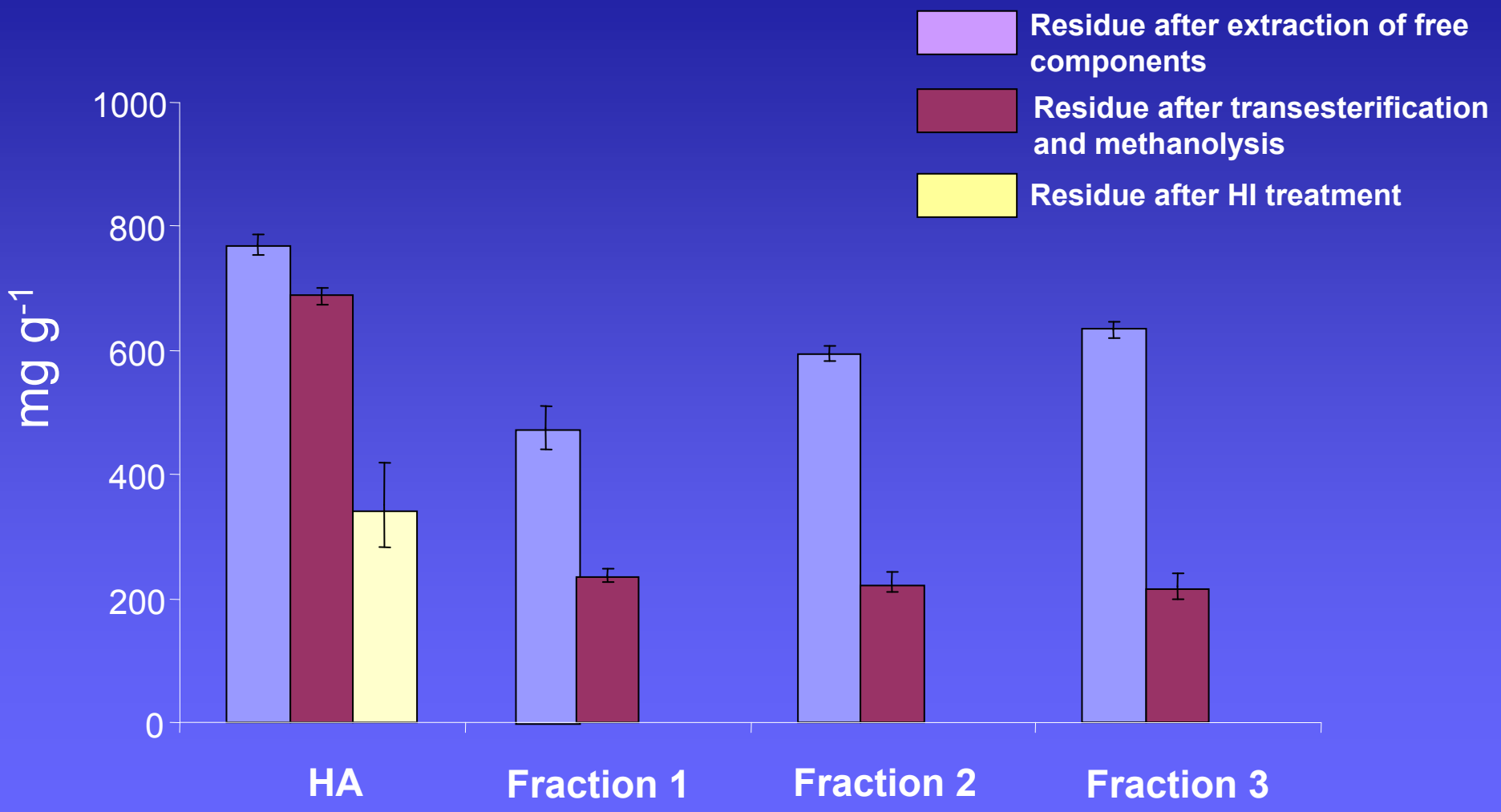


Total Yields (mg g^{-1}) of Organic Extracts by GC/MS

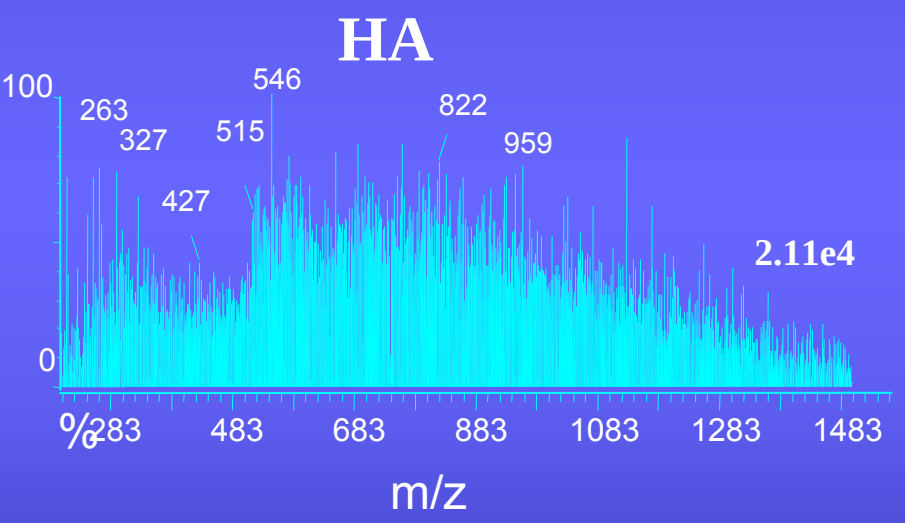


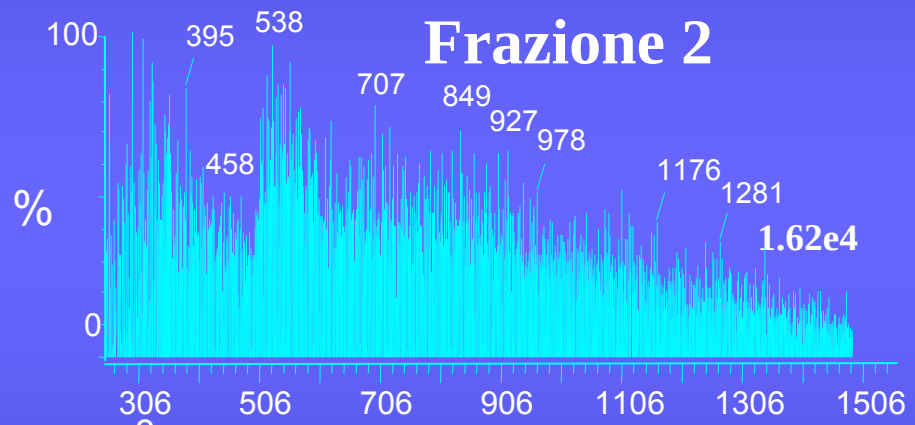
Total Yields (mg g^{-1}) of Components in Aqueous Phases (w/w)

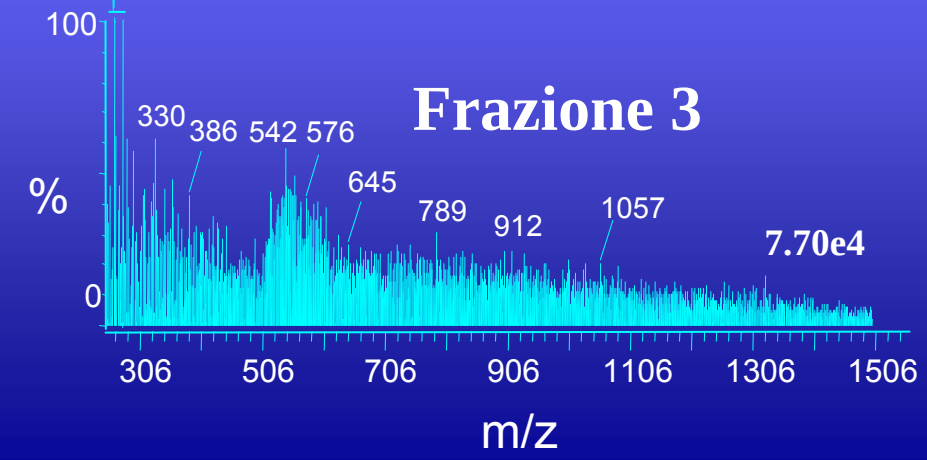
Distribution (mg/g) of solid residues resulting from various steps of sequential fractionation (w/w)



ESI-MS Spectra of HA and HPSEC size-fractions







THE SUPRAMOLECULAR NATURE OF HUMIC SUBSTANCES CAN BE EXPLOITED IN DIFFERENT WAYS:

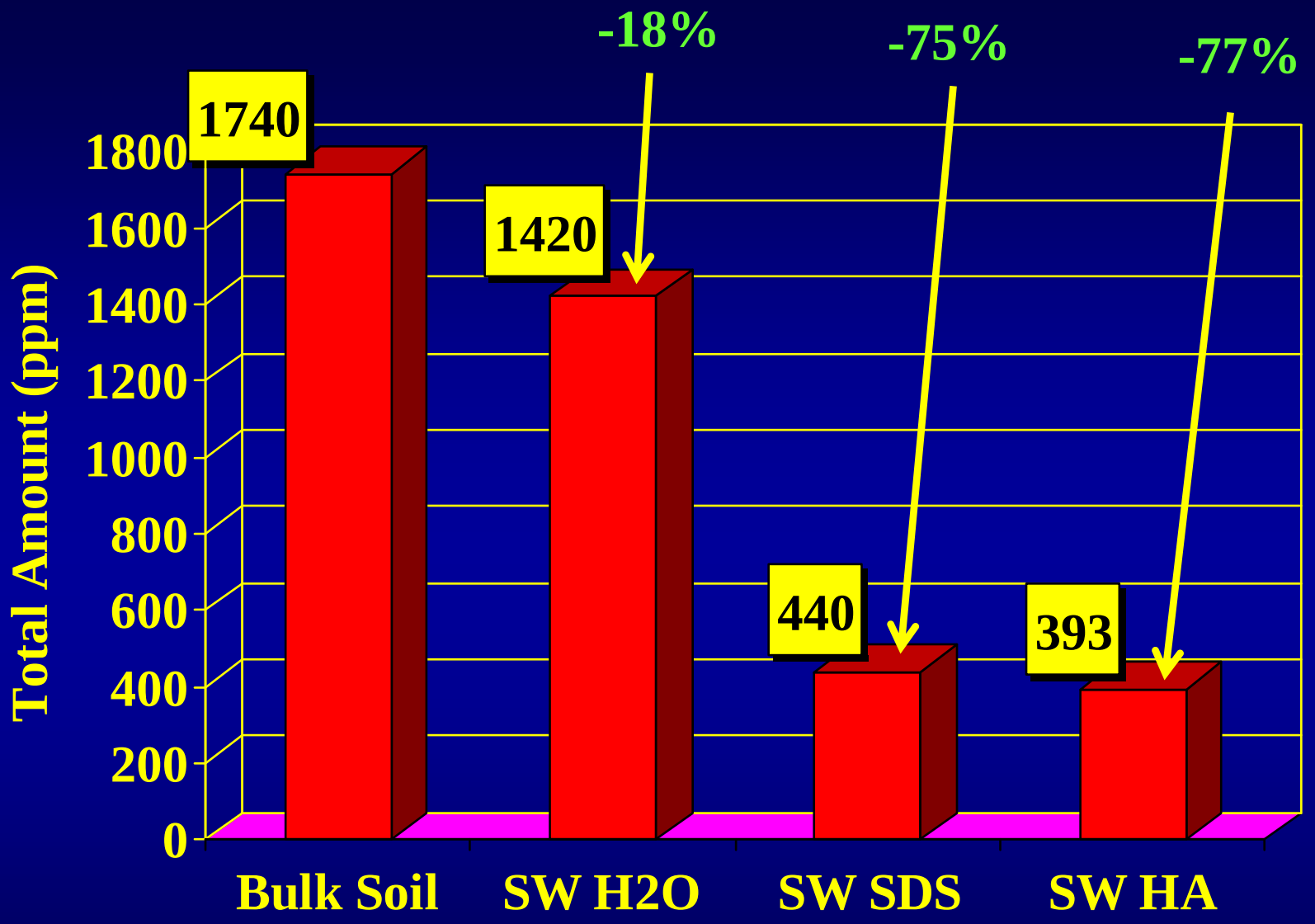
3. Fast, cheap, and eco-compatible remediation of contaminated soils by partitioning of pollutants in humic supramolecular structures

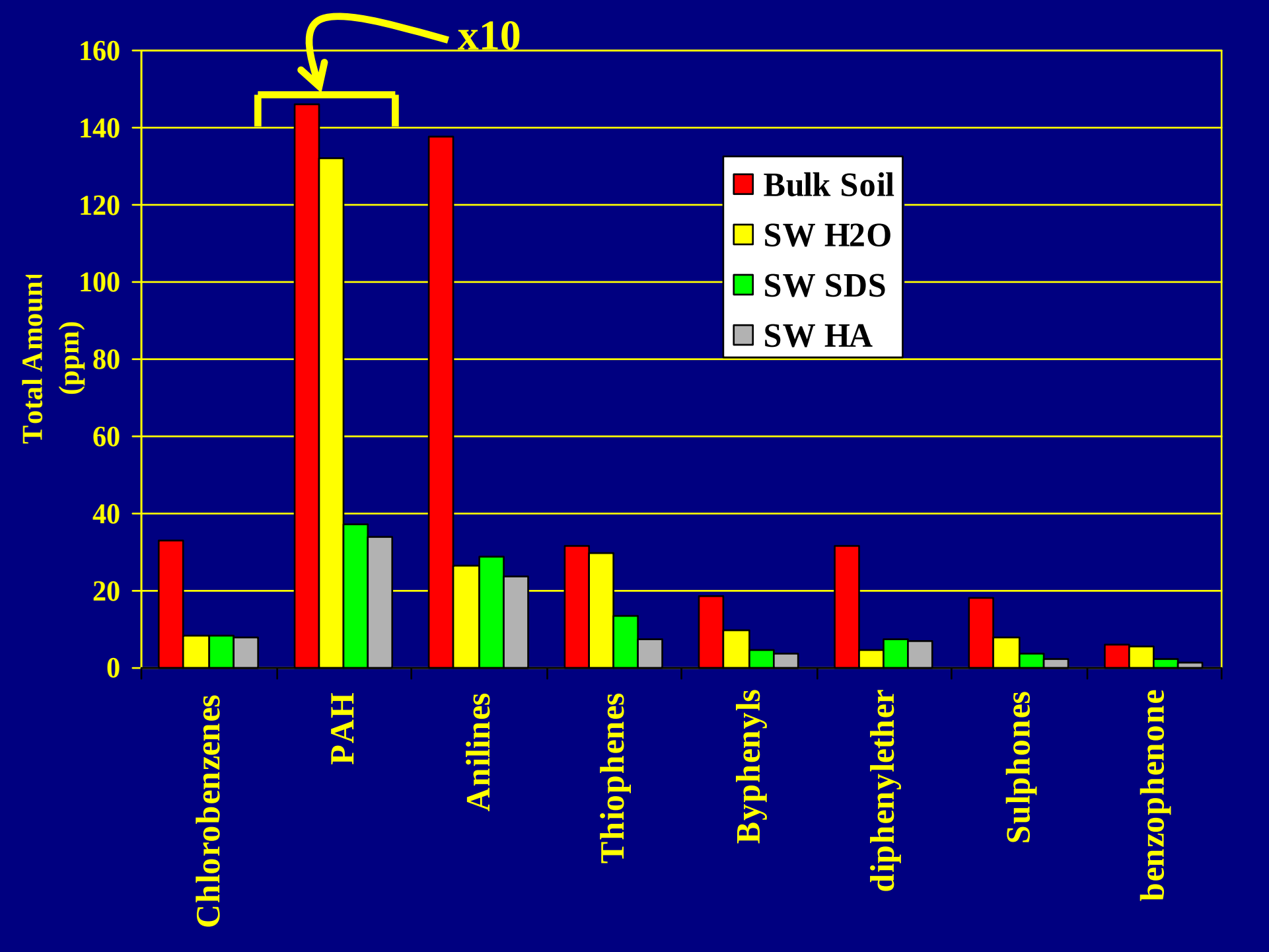
A polluted silty soil (A5.136B ACNA) was sampled from the heavily polluted ACNA industrial site at Cengio (Sv) in northern Italy

The extraction capacity of pollutants in water, SDS, and a Lignite HA was compared to suggest the best soil washing technique

Octafluoronaphtalene was used as internal reference for GC-MS quantification

Tr (min)	Sample
24.61	Trichlorobenzene (1,3,5 o 1,2,3)
24.87	Naphtalene
28.70	Tetrachlorotiofene
30.11	2-chloro-5-metyl aniline
30.45	4-chloro-2-metyl aniline
31.71	1,2,4,5-tetrachlorobenzene
31.88	2,6-dichloroaniline
32.73	2,4-dichloroaniline
33.83	1,2,3,4-tetrachlorobenzene
33.93	byphenil
34.96	diphenylether
35.58	2,6-dichloro-3-metyl aniline
36.18	3,5-dichloroaniline
40.41	pentachlorobenzene
40.62	3,4,5-trichloroaniline
46.97	esachlorobenzene
49.67	phenanthrene
51.92	2,3,4,5,6-pentachloroaniline
54.40	diphenylsulfon
55.80	9.10-anthracenedione
57.18	1-phenylmetyl naphtalene
60.89	1-chloro-9,10-anthracenedione
63.76	N-pheyil-1-naphtalenammine
66.55	1-ammino-9,10-anthracenedione
67.21	1,1'-binaphtalene
70.59	1,2'-binaphtalene
70.79	7[H]-benz[DE]-anthracen-7-one
73.46	1-(2-naphtalenylmetyl)-naphtalene
73.65	3-(4-phenylphenyl)-benzophenone


-18%
-75%
-77%
1740
1420
440
393
1800
1600
1400
1200
1000
800
600
400
200
0
Total Amount (ppm)
Bulk Soil
SW H2O
SW SDS
SW HA


x10
160
140
120
100
80
60
40
20
0
Total Amount (ppm)
Bulk Soil
SW H2O
SW SDS
SW HA
Chlorobenzenes
PAH
Anilines
Thiophenes
Byphenyls
diphenylether
Sulphones
benzophenone

- **Soil washing with natural humic substances was as efficient as that performed with a common ionic surfactant (SDS)**
- **SW HA extracted 77% of total pollutants versus 75% extracted by SW SDS**
- **PAHs, thiophenes, sulphones and benzophenones were more efficiently removed by HA than by SDS**
- **chlorobenzenes, anilines, byphenyls, and ethers were removed by HA soil washing as efficiently as by SDS**

FUTURE PERSPECTIVES

The understanding of the real nature of Natural Organic Matter (NOM) opens up challenging research opportunities :

1. **characterization of soils on the basis of organic matter components ;**

2. **relating structure and conformation of NOM to plant physiological and biochemical activity.**

3. **develop technologies to improve remediation of soil contaminants and control CO_2 emissions from soils.**

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Daniela Smejkalova (PhD, 2006)

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