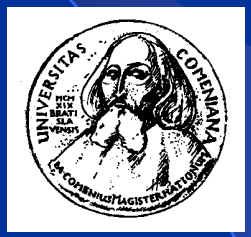
ANALYSIS AND CHARACTERIZATION OF HUMIC SUBSTANCES BY HPLC METHODS

Milan Hutta, Róbert Góra, Mária Chalányová, Radoslav Halko

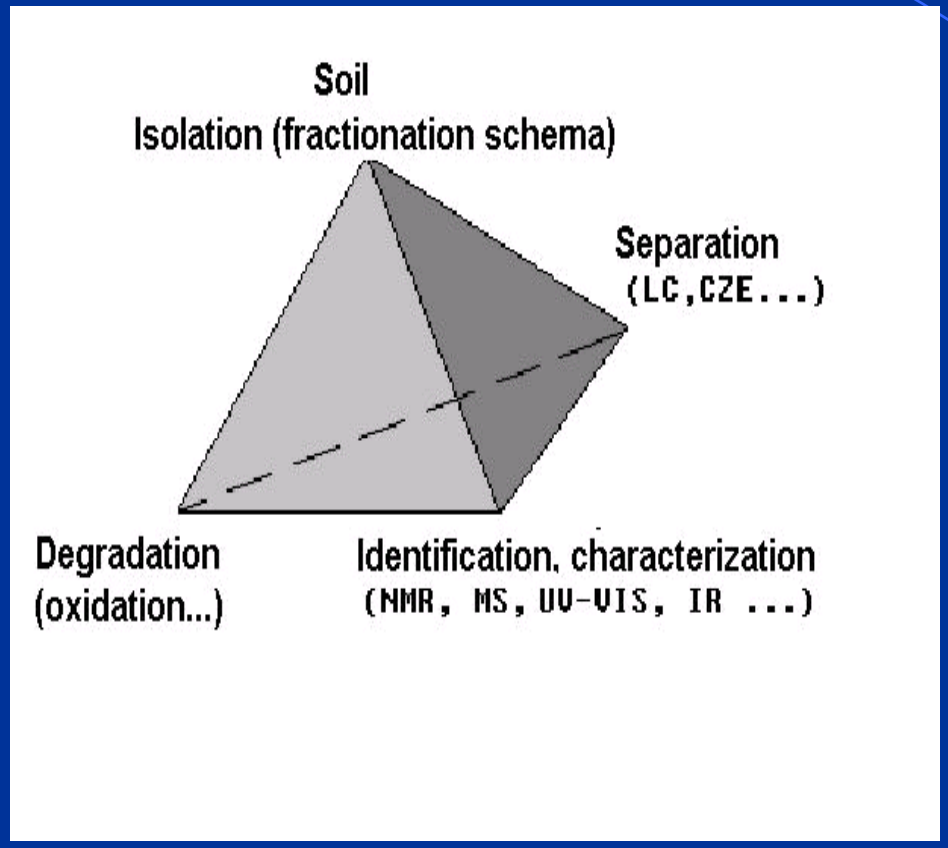
Department of Analytical Chemistry, Faculty of Natural Sciences,
Comenius University



Mlynska dolina CH-2, 842 15 Bratislava, Slovakia
hutta@fns.uniba.sk

APVV-0595-07, VVCE-0070-07, VEGA-1/4474/07, VEGA-1/0870/09

Introduction



Multicomponent analysis of ionogenic compounds complex environmental (humic substances, etc.) or biological matrices (biopolymers, low MW substances) is in many aspects still problematic, especially from the point-of-view of refined requirements of praxis.

Systematic combination of various separation principles and/or column separation methods hyphenation offers many attractive features of manipulation with selectivity of separation and detection and speed of analysis

Relation of sample complexity and theory of peak overlap in separation systems.

No one current single analytical method can resolve all components of

complex natural or technological samples as follows from

Davis and Giddings statistical peak overlap theory based on homogeneous statistics

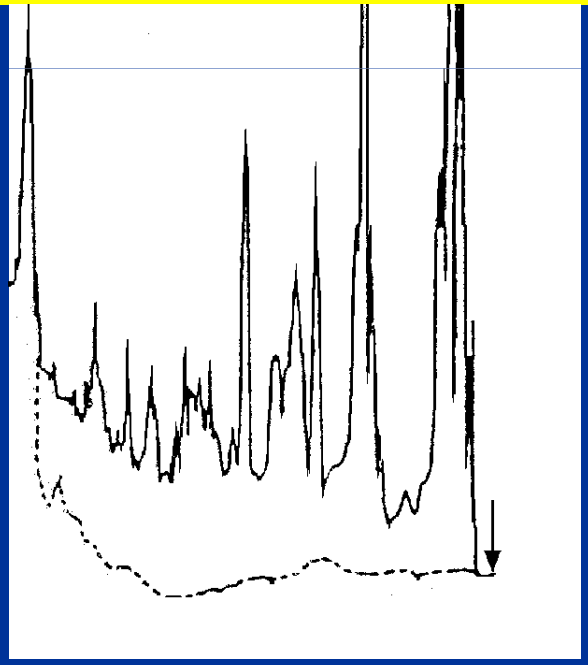
Davis J.M., Giddings J.C.: AnalChem., 55 (1983) 418.

J.M.Davis: Anal.Chem., 69 (1997) 3796.

What does it mean complex sample?

Each sample is complex at certain – usually low concentration level of its components when number of sample components exceeds component capacity of given separation or detection method. Soil column chromatography.

(even highly purified water or rain water are complex samples at ppt and lower levels).



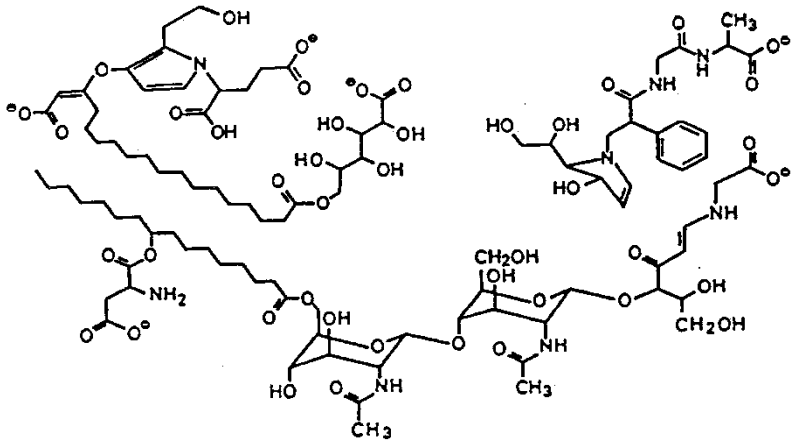
>10 ppm

1 ppm

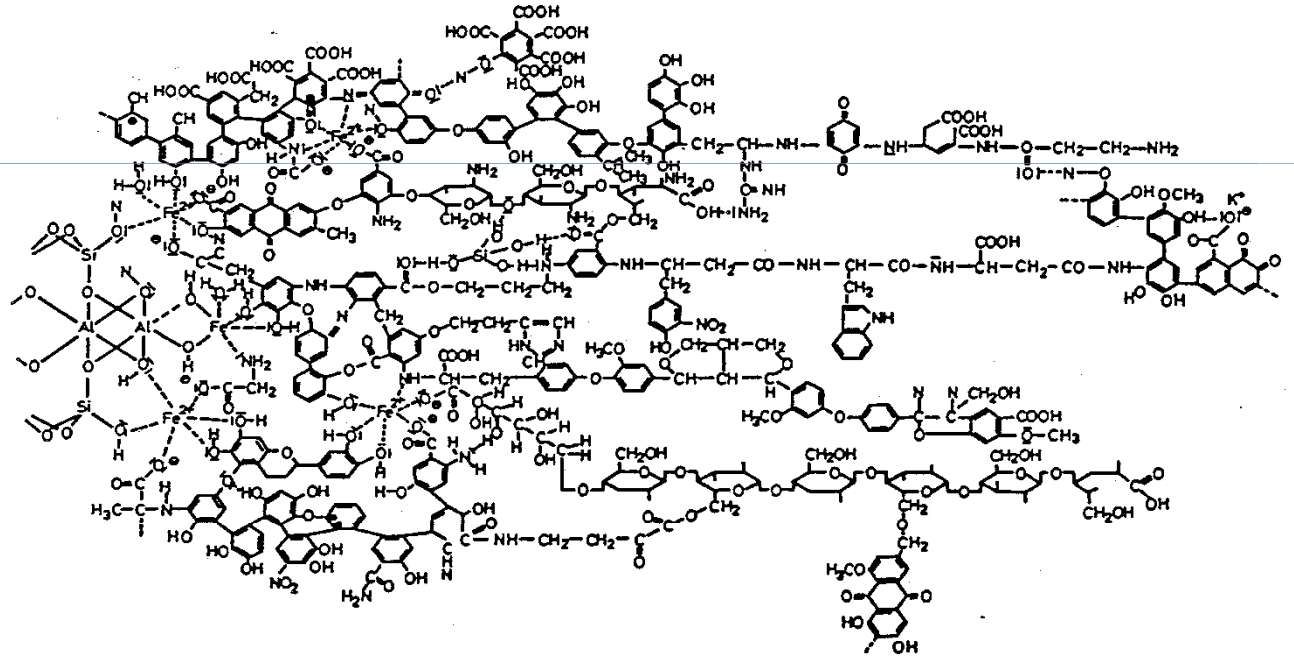
<0.1 ppm

Urine or manure RP-HPLC profile

(a)

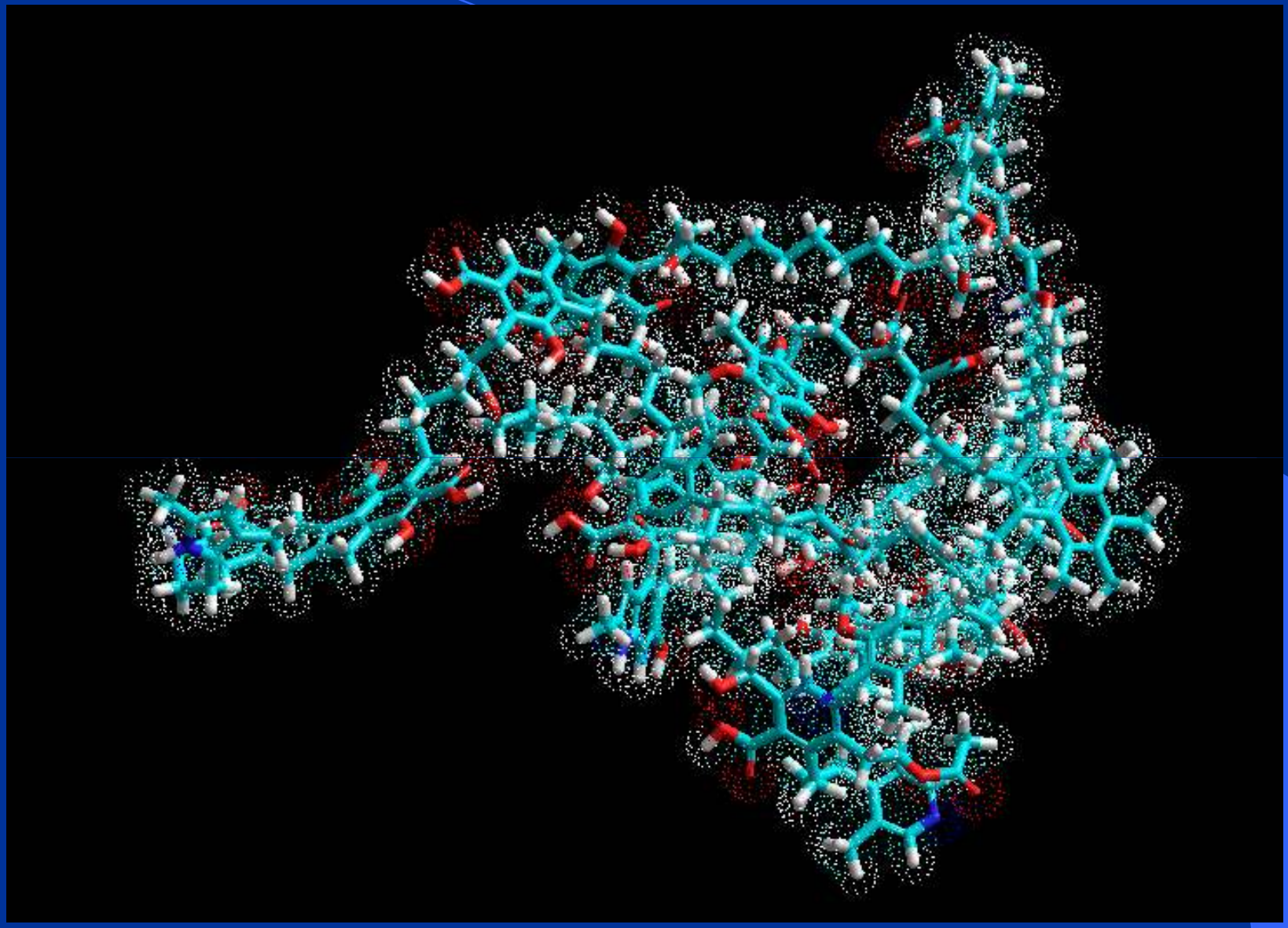


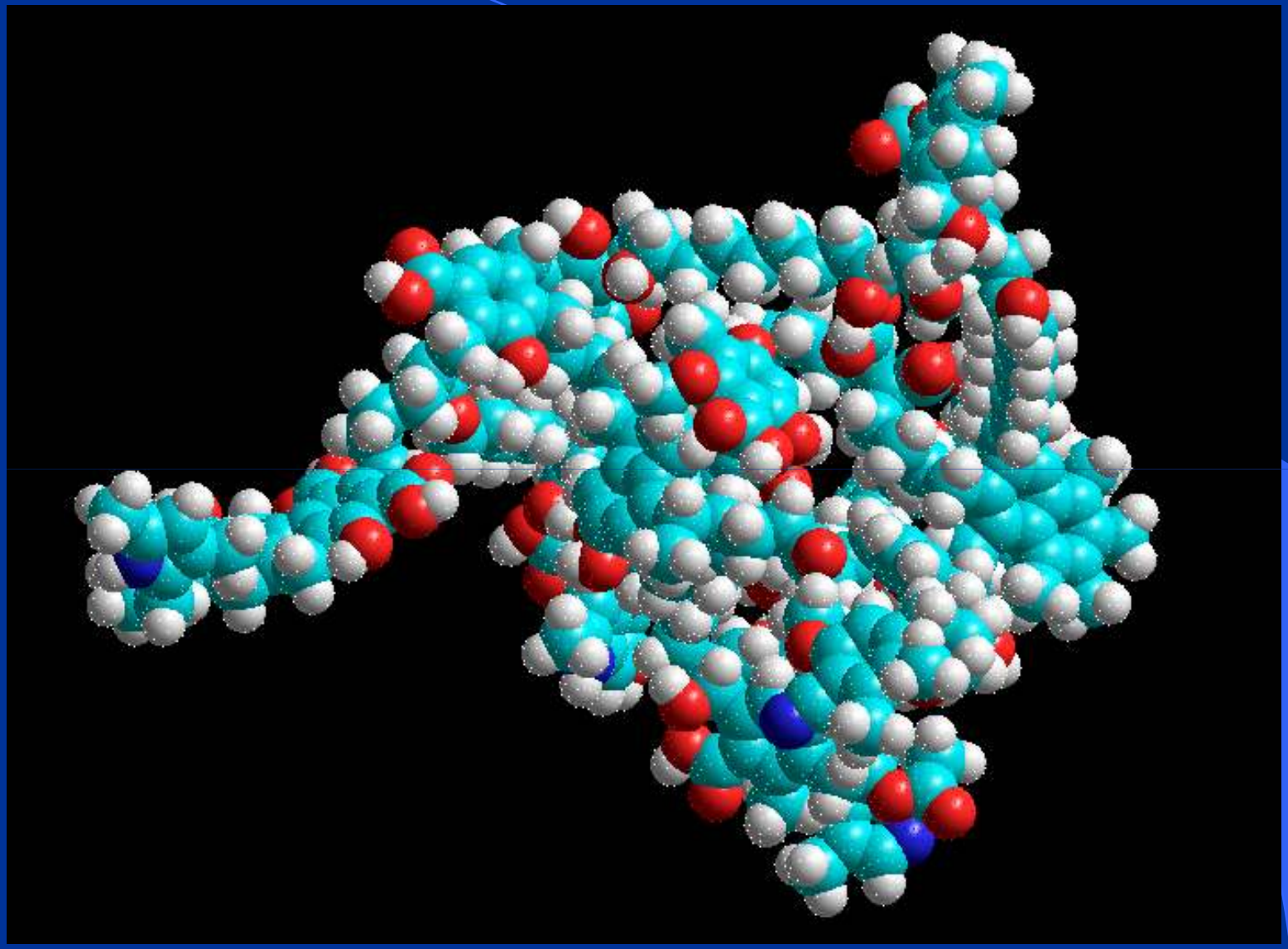
(b)



Models of aquogenic refractory organic matter (a) AROM: [34] and pedogenic refractory organic matter (b) PROM: [33].

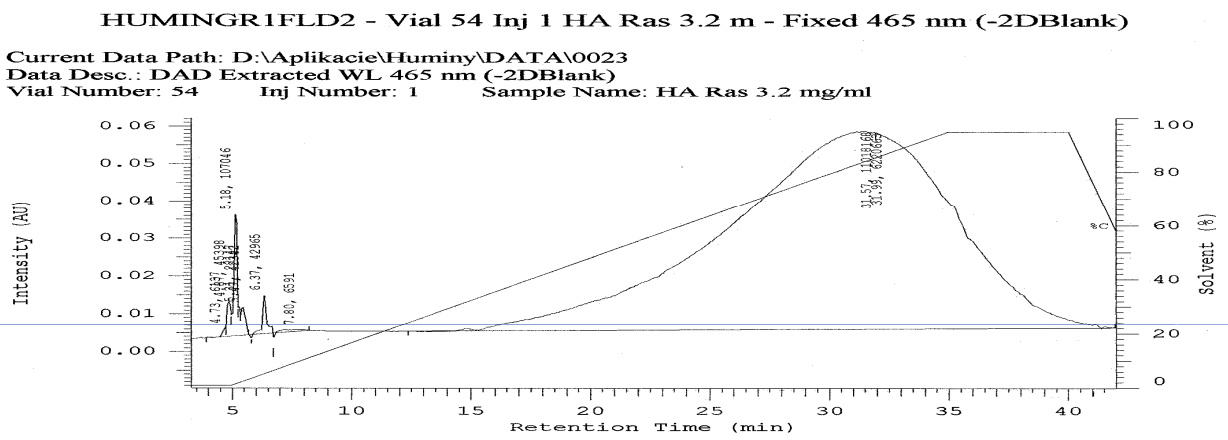
D. Kleinhempel, *Albrecht Thaer Archiv*, **14**, 3 (1970).
R. B. Gagosian and D. H. Stuermer, *Marine Chem.*, **5**, 605 (1977).

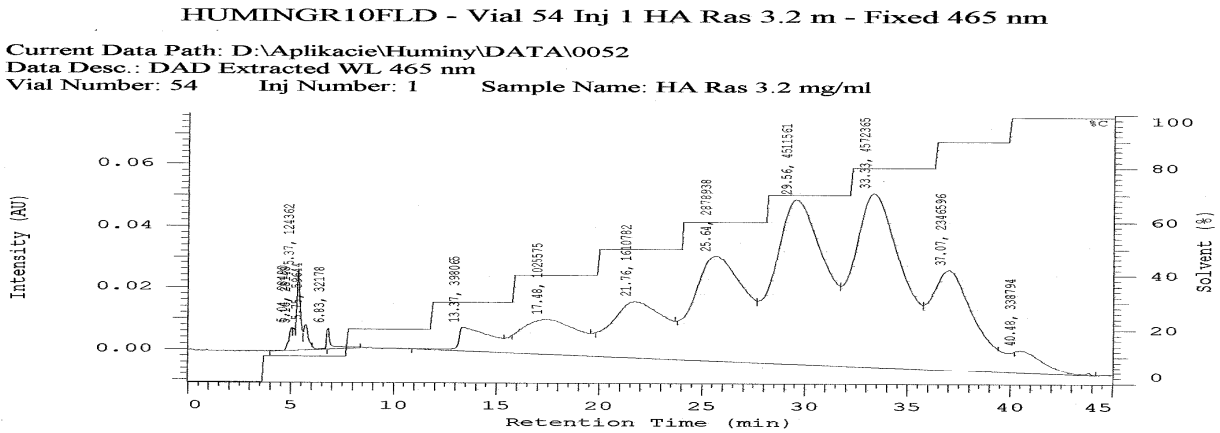




RP-HPLC profiles of Peat Humic Acid on 30 nm pore diameter C18 column

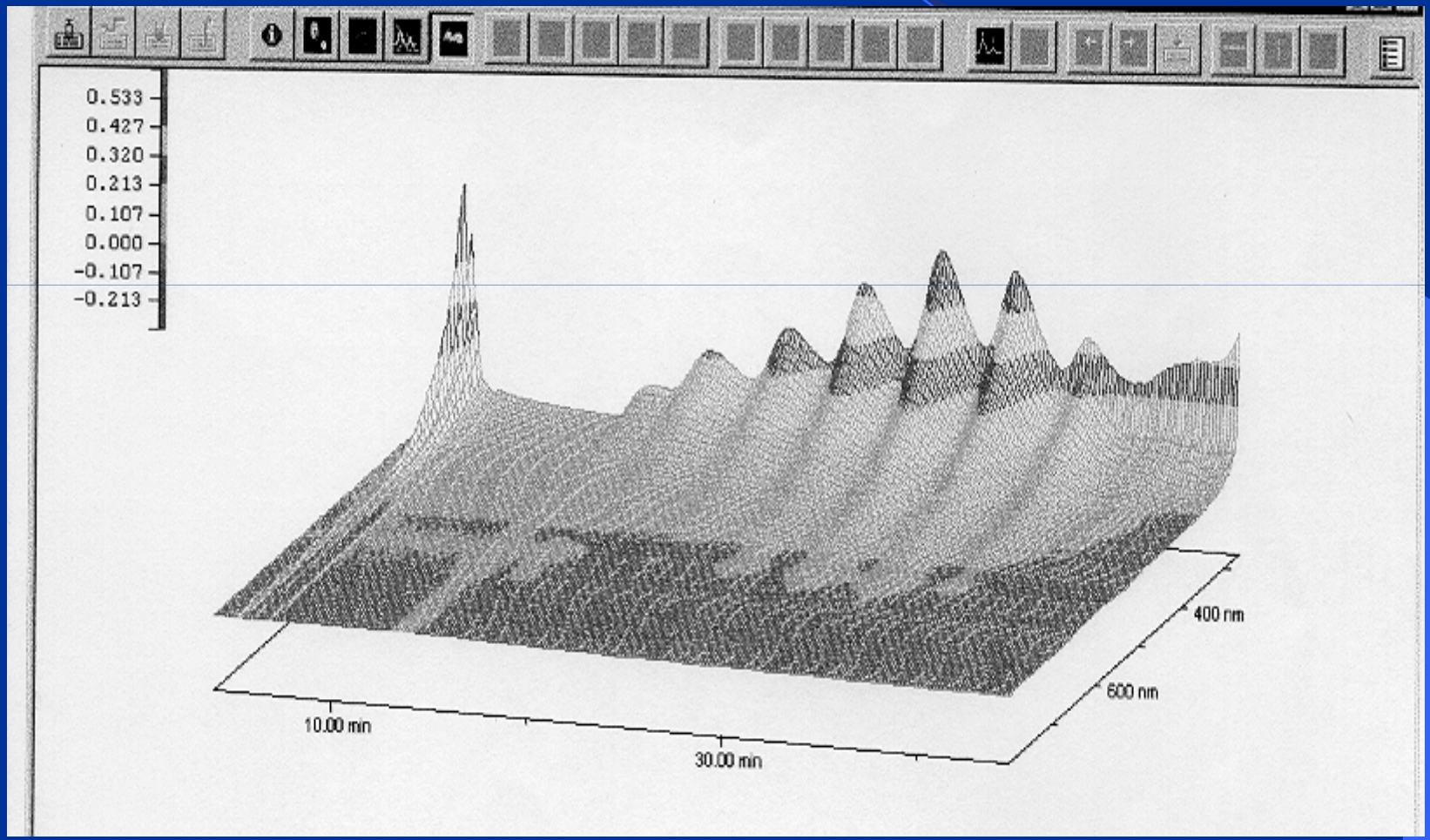
Note: similar profiles are typical for almost every analytical method





RP-HPLC profiles of Peat Humic Acid on 30 nm pore diameter C18 column

Note: similar profiles are typical for almost all analytical methods



Basic aspects of combination of liquid chromatography methods or alternative methods

For real sample pretreatment and multicomponent (trace) analysis we should achieve whenever possible

- high selectivity of redistribution (HPLC-variable modes) and detection
- high efficiency of transport phenomena (quantitative) and detection yield (sensitive, low LOD, LOQ)
- high enrichment (10^2-10^6) and pre-concentration factors achievable in gradient and column switching HPLC
- quantitative transfer of sample within analytical procedure and/or method
- accurate results
- short analysis duration (fast and ultrafast LC 1-10 min)
- low cost of analysis

This is practically impossible without complex and complicated analytical procedure schema, smart and expensive instrumentation and consistent analyses quality control.

Definite effort is given to elaboration of robust, validated and universally applicable analytical methods based also on HPLC.

In spite of fact that

system of quality assurance and quality control

suffers from

lack of proper certified HSs standard reference materials
refering to certain exact locality

for validation of both existing and new analytical methods

COMBINATION OF SEPARATION TECHNIQUES FOR ANALYSIS OF IONOGENIC SUBSTANCES IN COMPLEX MATRICES

Complexity of problems associated with **trace or ultratrace analysis (ppb to ppt)** of substances in a natural matrices is reflected mainly by the fact that

for their successful solution **we need to combine or hyphenate even highly efficient techniques.** Beside on-line coupled separation and identification or detection methods, e.g., HPLC-MS etc., possibilities of combinations and hyphenation of separation methods is intensively investigated and researched.

This originates so called two- or multi-dimensional separation techniques, e.g. HPLC-HPLC or ITP-CZE and the other.

These methods are according to their principles applicable for distinct groups of substances having differing numbers of entities.

By the combination of analytical methods we can according to the nature of problem and aim of the problem solution achieve that number of analysed substances:

- **increases** for methods complementary each other (some LC-LC methods, e.g. SEC- RPLC)
- **stays unchanged**, but the combination enables better characterization of sample and/or identification ability of sample components (e.g. PyGC-MS, IEX-RPLC-MS etc.) by increase of information content of analysis,
- **decreases** when such methods are combined, that first method has narrower applicability than the second one, but their combination gives higher discrimination ability.

CONCEPTS OF METHODS AND TECHNIQUES COMBINATION

Systematic change of selectivity during separation process enables us to attain practically not restricted separation possibilities under the condition that we are not restricted by available time.

Advantages of successive (tandem) use of various separation modes (mode sequencing) we can express mathematically. According to Giddings is **peak capacity of simple isocratic mode** ϕ given by equation:

$$\phi = 1 + (N^{1/2} / m). \ln(1 + k'_n)$$

where for unity resolution m = 4 (4 σ separation),
N = plate number,
k'_n = retention factor of last peak
in a series of peaks numbered from zero (nonretained) to n (last detectable peak).\

. Peak capacity ϕ is based on determination of maximal number of solutes (peaks), that can be resolved with unity resolution R_S = 1 in a given system and within certain time interval

Peak capacity ϕ

HPLC gradient elution	**200 - 300**
HPLC isocratic elution	**cca 50**
SEC isocratic elution	**3-10**
CZE	**200 – 800**
ITP	**cca 100**
IEF	**200 – 300**

If peak capacity of all n modes is equal, we get:

$$\phi_T = \phi^n$$

Requirement of non-redundancy, or non-correlation and orthogonality between individual combined modes is the main requirement and at the same time also assumption or premise of the relation

$$\phi_T = \phi^n$$

because on contrary

Combination of n equal modes(identical columns) in a series increases overall efficiency according to equation:

$$\phi_T = 1 + [(nN)^{1/2} / m]. \ln(1 + k_n)$$

what is approximately equal to

$$\phi_T \sim n^{1/2} . \phi_i$$

Requirement of non-redundancy, or non-correlation and orthogonality

Orthogonality of separations or detection is a discussed philosophy of multi-dimensional system design.

Orthogonal design means that features multiply each other's utility, and overlap as little as possible.

Given set of carefully-crafted orthogonal features can enable a wide variety of possibilities for complex analysis through combining those features.

In theory, this means that one can have a small and simple instrumental base, which enables an extremely rich methodological environment for chemical analysis.

The highest separation efficiency is achieved by discrete combination of selective methods.

For proper selection of the most effective combinations we should take into account that correlation of the data is not given only by physico-chemical principle of combined methods, but also by a sample itself

- **chemical structure of components**
- **number of sample components**

and favorites proper choice of

differently selective – focused to dissimilar sample properties,
high performance and
very compatible separation processes.

Multicolumn switching Liquid Chromatography
Liquid Chromatography and Capillary Electrophoresis

Criteria for selection of methods suitable for combining in analytical separation schema applied to methods of liquid chromatography and capillary electrophoresis

Efficiency, Selectivity, Compatibility considerations

HPLC

Efficiency 1000 to 10000 plates per column. Exceptionally 1 - 2 orders of magnitude higher

Peak capacity – for common standard columns (250mm/4mm,3-5μm) isocratic 50-100, gradient 250-300, SEC 5 to 20

CZE

Efficiency 5000 to 500 000, peak capacity 300 - 500 for low molecular weight substances and 500 to 800 for polymeric substances.

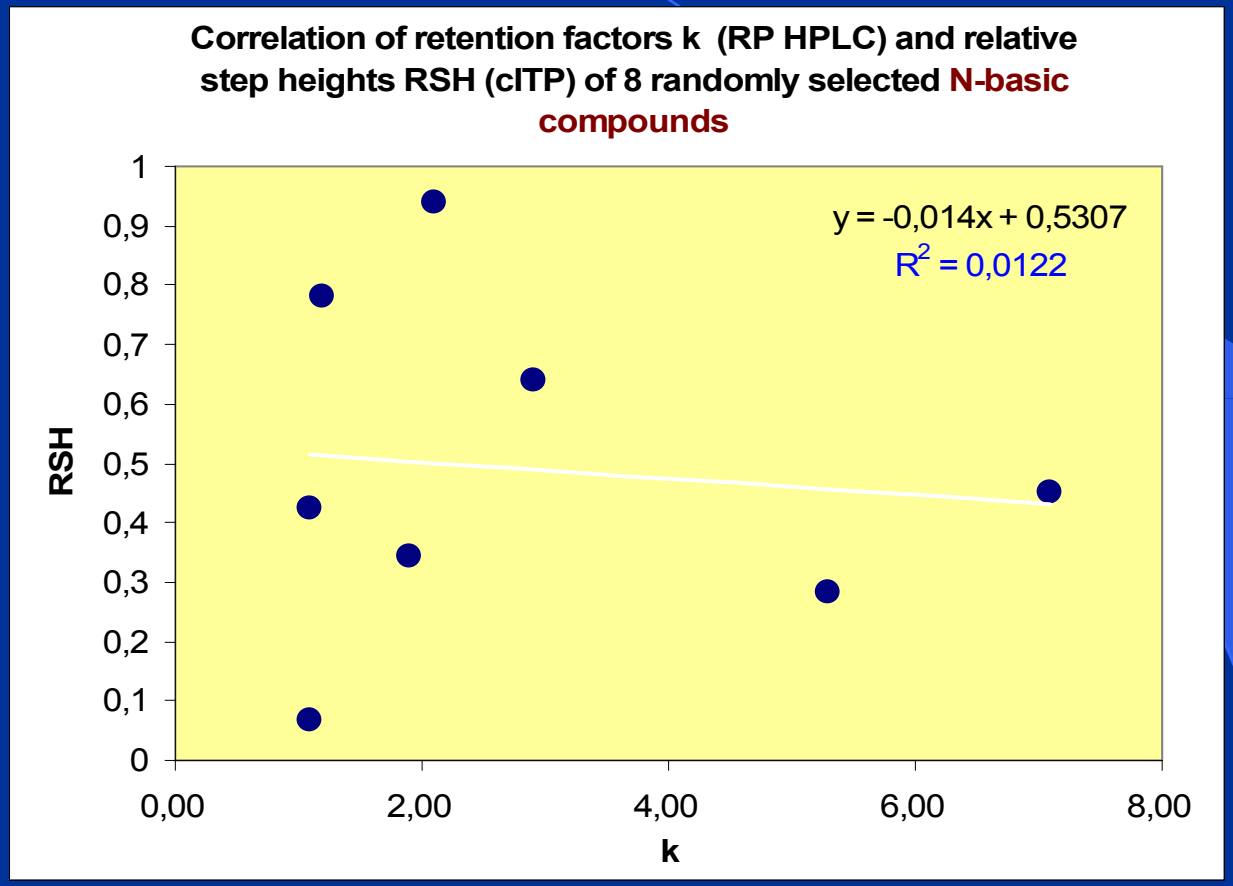
ITP

peak capacity equivalent -"zone capacity" is estimated to **50 - 100** .

Search for orthogonal combinations of RPHPLC and cITP

Selectivity

Compound	RSH
o-toluidine	0,9381
aniline	0,7797
p-toluidine	0,6384
2,4,6-trimethylpyridine	0,4521
triethylamin	0,4237
pyridine	0,3446
2-aminopyridine	0,2825
diethylenetriamine	0,0678



Separation conditions:

HPLC - Separon SIX C18, CGC 5um, MeOH / H_2O

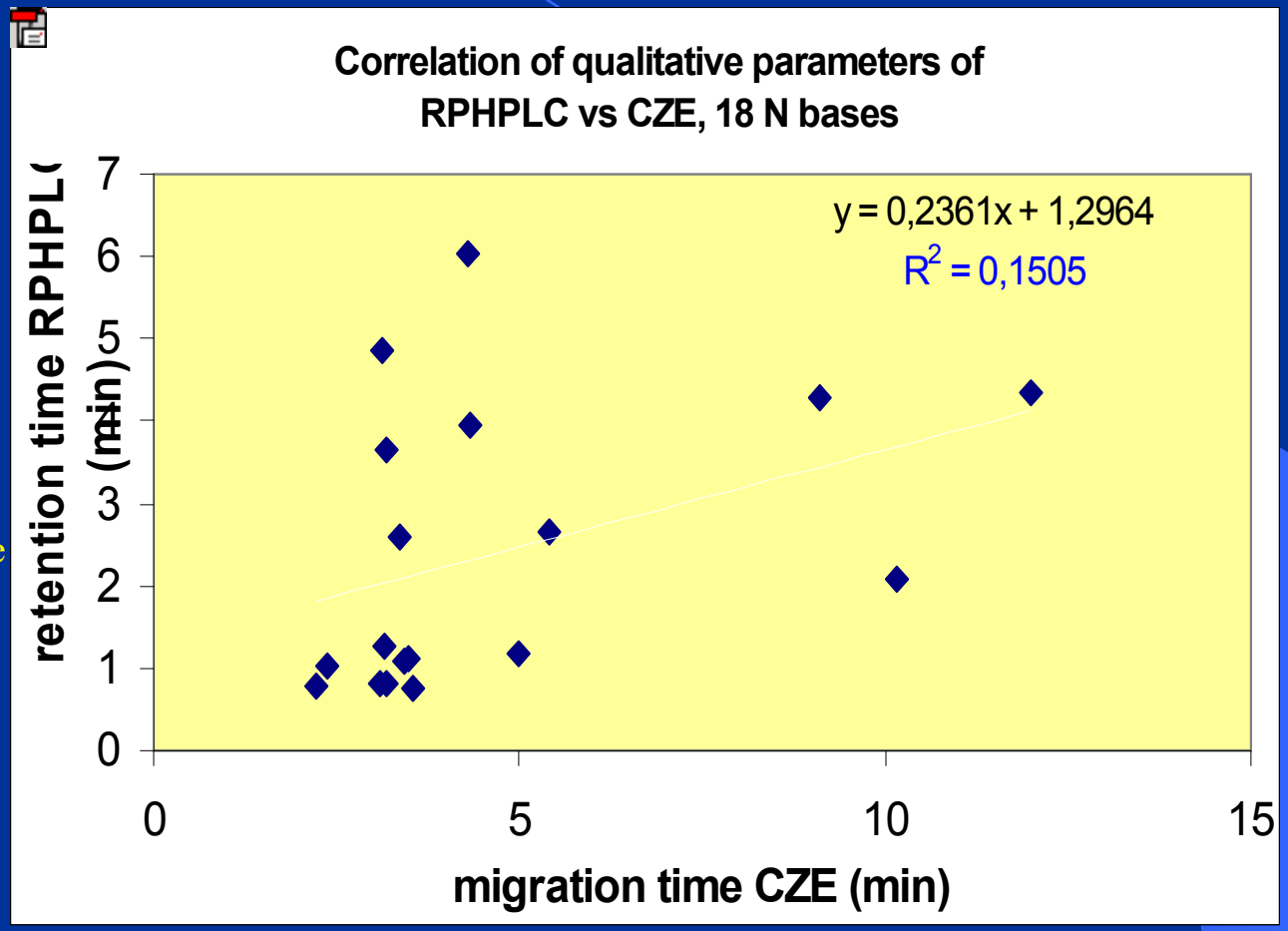
cITP- LE: ammonia 10mM , acetate 15mM, HEC, pH=5,2
TE: acetic acid 10 mM, pH=5

Hutta M., Kaniansky D., Zelenska V. unpublished results

Search for orthogonal combinations of LC and CE

Selectivity at High Compatibility

imidazole
pyridine
creatinine
quinine
aniline
trimethylpyridine
cytozine
o-toluidine
nicotinamide
adenine
histidine
N-1naphthylethylenediamine
4-aminophenazone
cytidine
adenosine
tryptophane
tyrosine
p-aminoacetophenone



Separation conditions:

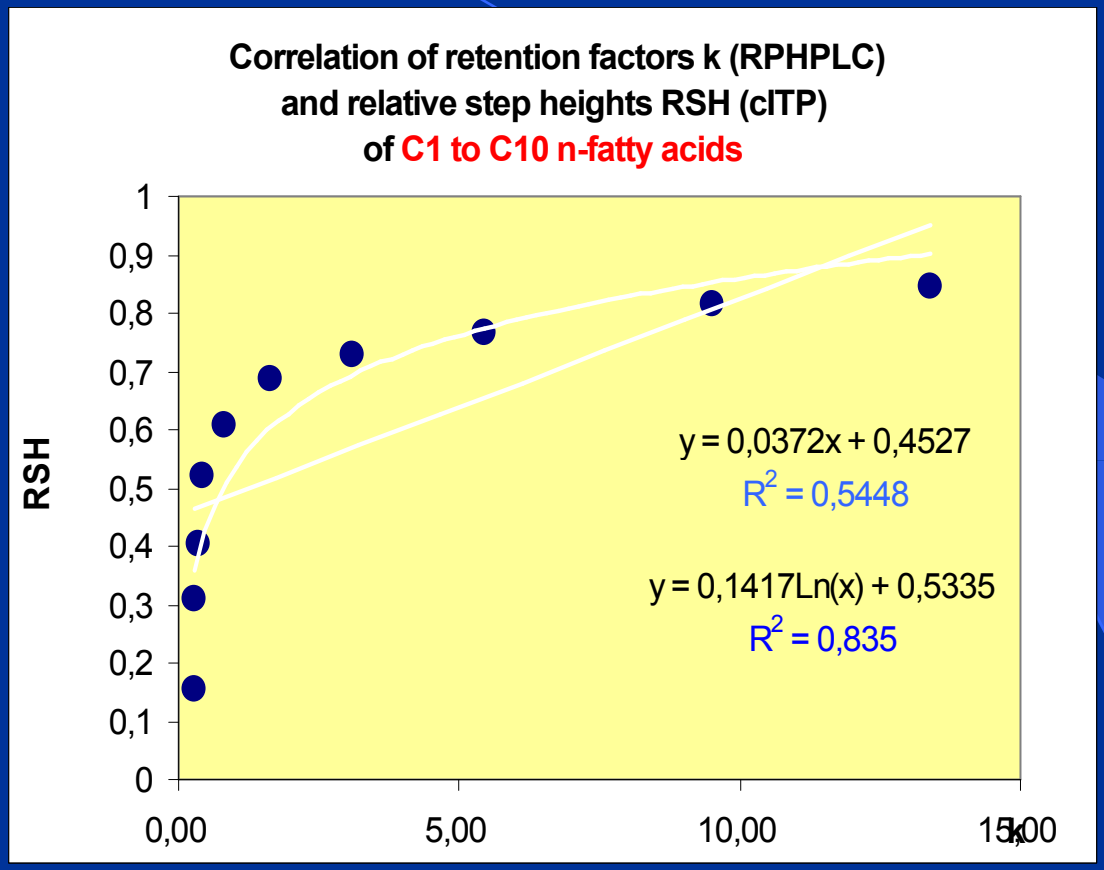
HPLC - Chromolith Performance RP18e, MeOH / pH 2.5 buffer CZE - background electrolyte , pH 2.5

Humic Substances in Ecosystems 8,
Soporna 13.-17.9.2009 Slovakia

Hutta M., Mamrošová M., Ondrejášová B . unpublished results

Search for orthogonal combinations of LC and CE
Selectivity

Compound	RSH
Formic acid	0,156
Acetic acid	0,311
Propionic acid	0,405
Butyric acid	0,522
Valeric acid	0,606
Hexanoic acid	0,685
Heptanoic acid	0,728
Octanoic acid	0,767
Nonanoic acid	0,815
Decanoic acid	0,844



HPLC Purospher RP C18e, Methanol-water, pH 2.0 ITP LE, TE

Hutta M., Kaniansky D., Kovaľ M., unpublished results

Discrete vs continual

In principle we can combine two methods as

- **<u>discrete methods</u>, 2D separation then exploit greater separation space in which every and each sample component has its own exact coordinates**

- **<u>continual methods</u>, 2D separation requires instant presence of both separation mechanisms and movement of separated components we can imagine as trajectories of points in 2D space, what restricts available separation space (2 D Planar LC, CEC, MEKC)**

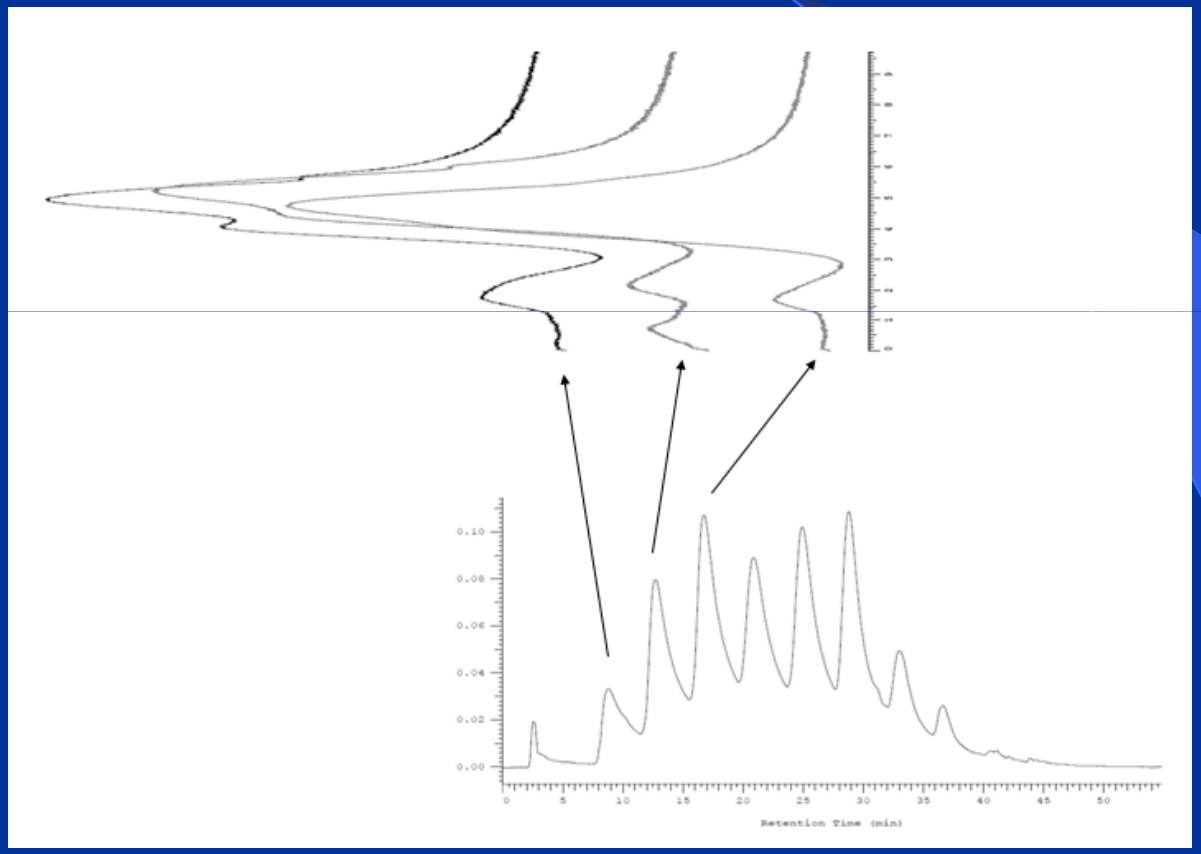
Compatibility and selectivity considerations – microbore, liquid (mobile, carrier, electrolyte) phase

HPLC	**HPLC**	**c ITP**	**CZE**
Diluting or concentrating	Diluting or concentrating	Concentrating	Diluting
Reversed Phase	Reversed Phase	pK	pK
Size exclusion	Size exclusion	Effective mobility in fully charged state	Effective mobility in fully charged state
Ion Exchange	Ion Exchange	Secondary equilibria	Secondary equilibria
HILIC	HILIC	Complexation	Complexation
Hydrophobic Interaction	Hydrophobic Interaction	Inclusion	Inclusion
Affinity IMAC	Affinity IMAC	Solvation	Solvation
Normal Phase	Normal Phase	Counterion	Counterion

ANALYSIS AND CHARACTERIZATION OF HUMIC SUBSTANCES ISOLATED FROM PEAT AND SOIL USING OFF-LINE COMBINATION OF LIQUID CHROMATOGRAPHIC METHODS

Róbert Góra, Pavol Rohárik, Milan Hutta

SEC, Separon HEMA 100, DMF/buffer pH 3.00



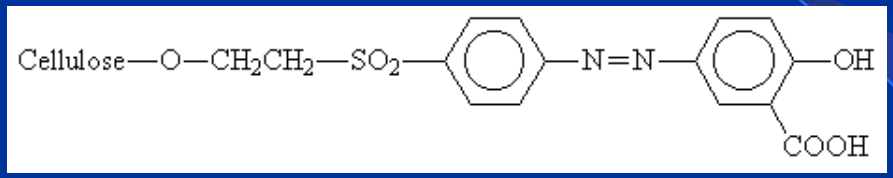
RP-HPLC C18, DMF/buffer pH 3.00

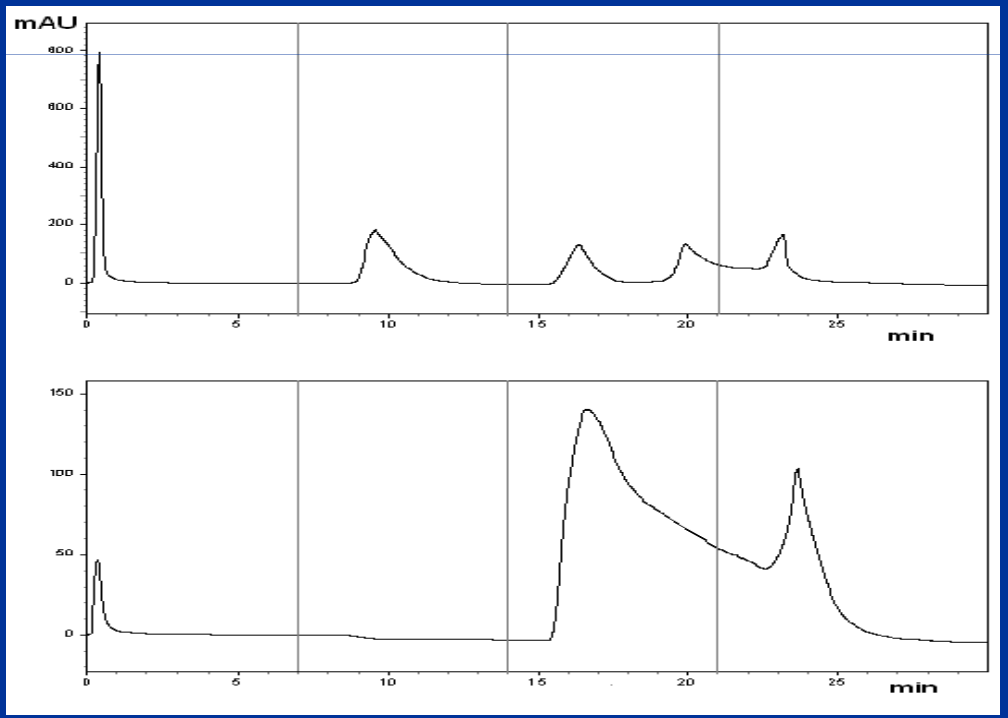
IMAC

UTILIZATION OF IMMOBILIZED ALUMINIUM(III) METAL ION AFFINITY CHROMATOGRAPHY FOR ANALYSIS OF HUMIC ACID

Radoslav Halko, Tibor Neuročný, Milan Hutta

Structure of chelate sorbent Iontosorb SALICYL – Al(III)





Coupling and Interfacing

Four levels of coupling can be distinguished :

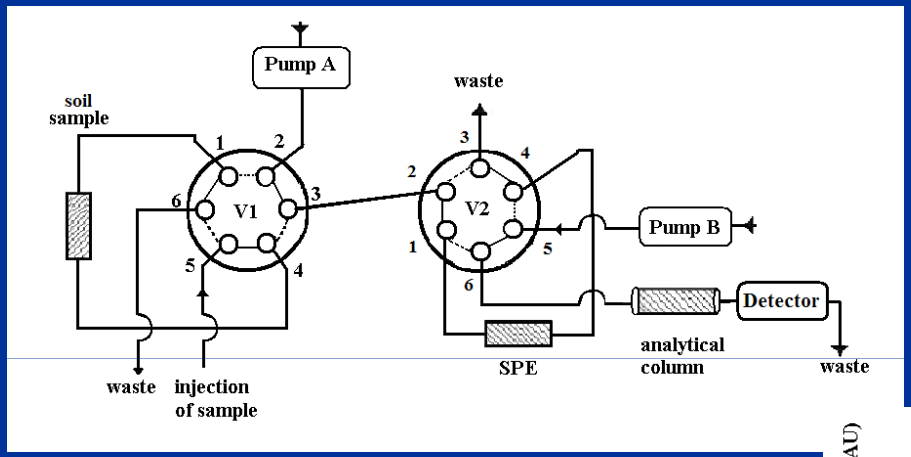
- off-line (manual),
- at-line (robotic interface)
- on-line (coupling via a transfer line)
- in-line (complete integration including sample preparation)

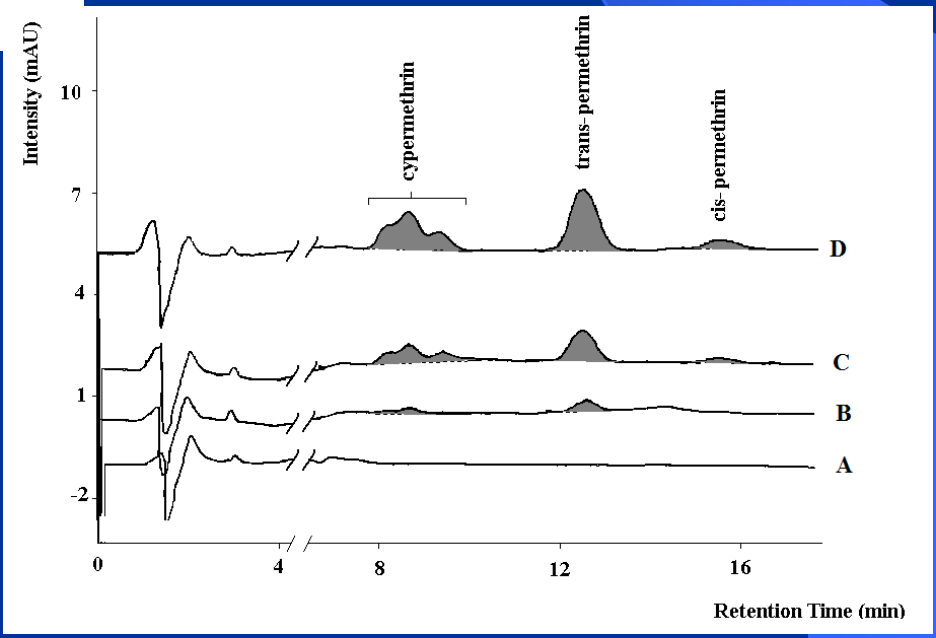
Heart-cutting (non-comprehensive) or comprehensive sampling from first to second step or dimension

Valcárcel M. et al. 2001

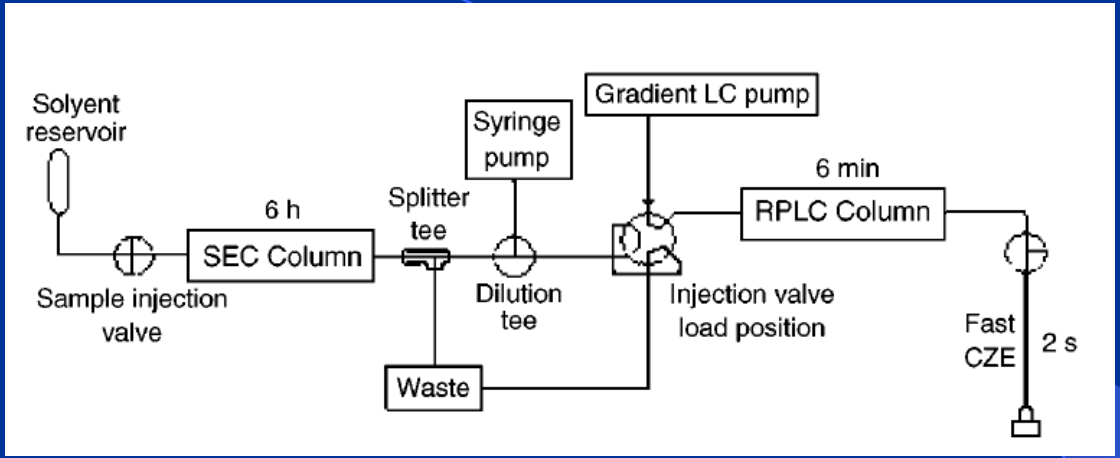
ON-LINE FLOW-THROUGH EXTRACTION–PRECONCENTRATION-LARGE VOLUME INJECTION REVERSED-PHASE LIQUID CHROMATOGRAPHY METHOD FOR TRACE DETERMINATION OF SELECTED PYRETHROIDS IN SLOVAK SOIL MICRO-SAMPLES

Mária Chalányová, Milan Hutta, Martin Pagáč





3 D
SEC (6 hours) – RPLC (6 minutes) – CE (2 seconds)



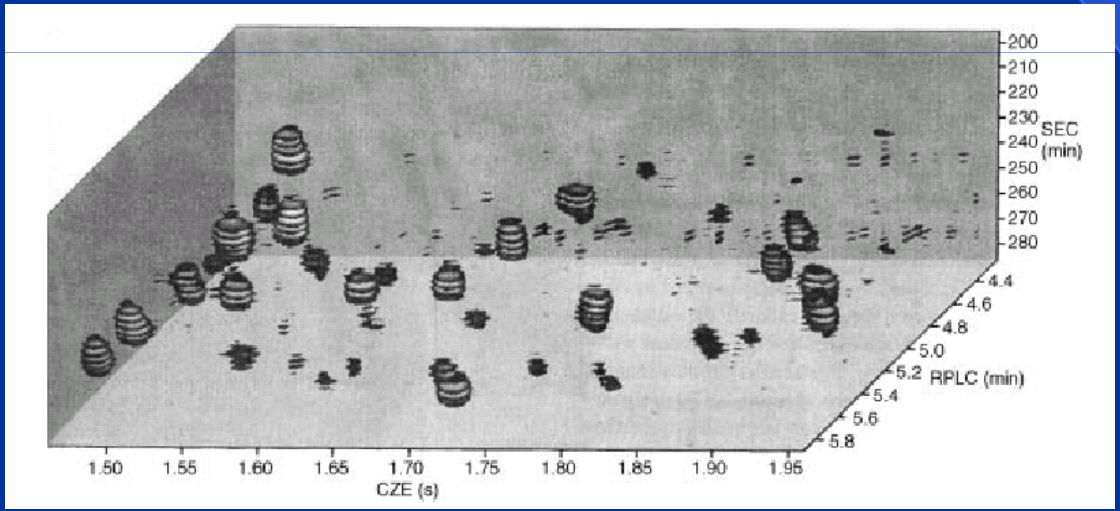


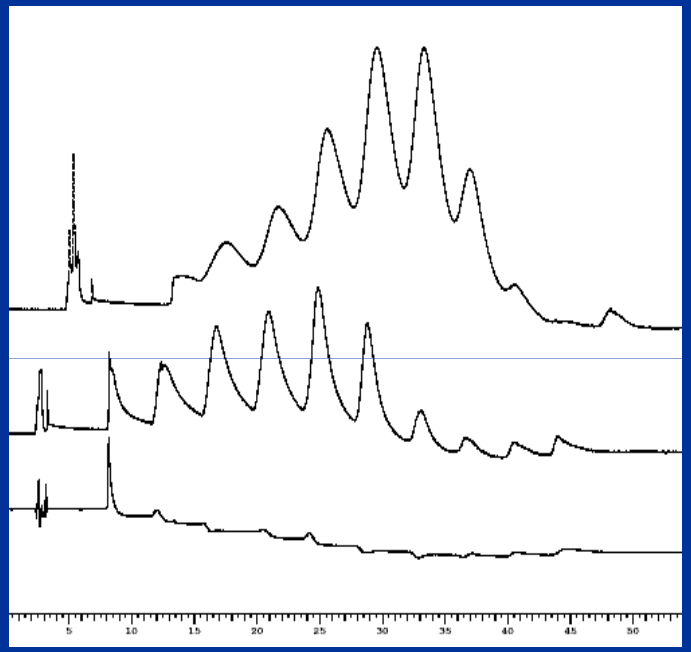
Figure 9.10 Three-dimensional representation of the data 'volume' of a tryptic digest of ovalbumin. Series of planar slices through the data volume produce stacks of disks in order to show peaks. Reprinted from *Analytical Chemistry*, **67**, A. W. Moore Jr and J. W. Jorgenson, 'Comprehensive three-dimensional separation of peptides using size exclusion chromatography/reversed phase liquid chromatography/optically gated capillary zone electrophoresis,' pp. 3456–3463, copyright 1995, with permission from the American Chemical Society.

Moore, Jorgenson 1995

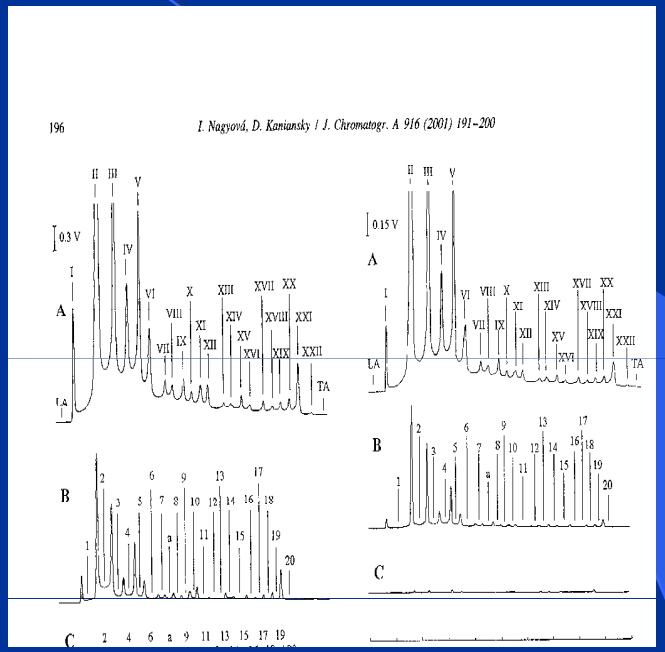
Interfacing HPLC and ITP - CZE

RP HPLC

c ITP



X



= ?

Hutta M., Gora R. 2003

Nagyova I., Kaniansky D., 2001

Orthogonal systems for humic substances characterisation ?

Future trends ?

- Search for orthogonal systems
- 3 D HPLC-HPLC-ITP-CZE – (DAD, 3D FLD, ELSD, EC Arrays, MSn…)
- Fast selectivity tuning 1-3 min per method

Past D Present DD Future DDD and more DDDD…