ANALYSIS AND CHARACTERIZATION OF HUMIC SUBSTANCES BY HPLC METHODS

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

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).

Urine or manure RP-HPLC profile

- >10 ppm
- 1 ppm
- <0.1 ppm

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

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.

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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.
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 $\phi$ given by equation:

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

where for unity resolution $m = 4$ (4 $\sigma$ 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 $\phi$ 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 $\phi$

<table>
<thead>
<tr>
<th>Method</th>
<th>Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPLC gradient elution</td>
<td>200–300</td>
</tr>
<tr>
<td>HPLC isocratic elution</td>
<td>cca 50</td>
</tr>
<tr>
<td>SEC isocratic elution</td>
<td>3–10</td>
</tr>
<tr>
<td>CZE</td>
<td>200–800</td>
</tr>
<tr>
<td>ITP</td>
<td>cca 100</td>
</tr>
<tr>
<td>IEF</td>
<td>200–300</td>
</tr>
</tbody>
</table>

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

$$\phi_T = \phi^n$$

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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:

\[ \phi_T = 1 + \left[ \frac{(nN)^{1/2}}{m} \right] \ln(1 + k_n) \]

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

<table>
<thead>
<tr>
<th>Compound</th>
<th>Abbrev</th>
<th>(k')</th>
<th>RSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>o-toluidine</td>
<td>OTO</td>
<td>2.10</td>
<td>0.9381</td>
</tr>
<tr>
<td>aniline</td>
<td>A0</td>
<td>1.20</td>
<td>0.7797</td>
</tr>
<tr>
<td>p-toluidine</td>
<td>PTO</td>
<td>2.90</td>
<td>0.6384</td>
</tr>
<tr>
<td>2,4,6-trimethylpyridine</td>
<td>TMP</td>
<td>7.10</td>
<td>0.4521</td>
</tr>
<tr>
<td>triethylamin</td>
<td>TEA</td>
<td>1.10</td>
<td>0.4237</td>
</tr>
<tr>
<td>pyridine</td>
<td>PYR</td>
<td>1.90</td>
<td>0.3446</td>
</tr>
<tr>
<td>2-aminopyridine</td>
<td>APY</td>
<td>5.30</td>
<td>0.2825</td>
</tr>
<tr>
<td>diethylenetriamine</td>
<td>DEA</td>
<td>1.10</td>
<td>0.0678</td>
</tr>
</tbody>
</table>

Correlation of retention factors \(k\) (RP HPLC) and relative step heights \(RSH\) (cITP) of 8 randomly selected N-basic compounds:

\[
y = -0.014x + 0.5307 \\
R^2 = 0.0122
\]

Separation conditions:
- **HPLC**: Separon SIX C18, CGC 5um, MeOH / H2O
- **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

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Search for orthogonal combinations of LC and CE

Selectivity at High Compatibility

Correlation of qualitative parameters of RPHPLC vs CZE, 18 N bases

\[ y = 0.2361x + 1.2964 \]
\[ R^2 = 0.1505 \]

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

Hutta M., Mamrošová M., Ondrejášová B. unpublished results
Search for orthogonal combinations of LC and CE Selectivity

<table>
<thead>
<tr>
<th>Compound</th>
<th>C1</th>
<th>C2</th>
<th>C3</th>
<th>C4</th>
<th>C5</th>
<th>C6</th>
<th>C7</th>
<th>C8</th>
<th>C9</th>
<th>C10</th>
<th>RSH</th>
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<tbody>
<tr>
<td>Formic acid</td>
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<td>0,156</td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
<td>0,156</td>
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<tr>
<td>Acetic acid</td>
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<td>0,311</td>
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<td>Propionic acid</td>
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<td>0,405</td>
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<td></td>
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<td>Butyric acid</td>
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<td>0,522</td>
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<td>Valeric acid</td>
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<td>0,606</td>
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<td>Hexanoic acid</td>
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<td>0,685</td>
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<td>0,685</td>
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<tr>
<td>Heptanoic acid</td>
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<td>3,11</td>
<td>0,728</td>
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<td>0,728</td>
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<tr>
<td>Octanoic acid</td>
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<td></td>
<td>5,44</td>
<td>0,767</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>0,767</td>
</tr>
<tr>
<td>Nonanoic acid</td>
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<td></td>
<td>9,50</td>
<td>0,815</td>
<td></td>
<td></td>
<td></td>
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<td>0,815</td>
</tr>
<tr>
<td>Decanoic acid</td>
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<td>13,40</td>
<td>0,844</td>
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<td></td>
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<td>0,844</td>
</tr>
</tbody>
</table>

Correlation of retention factors $k$ (RPHPLC) and relative step heights $R_{SH}$ (cITP) of C1 to C10 n-fatty acids

$y = 0.0372x + 0.4527$  
$R^2 = 0.5448$

$y = 0.1417\ln(x) + 0.5335$  
$R^2 = 0.835$

HPLC Purospher RP C18e, Methanol-water, pH 2.0

ITP LE, TE

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

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Discrete vs continual

In principle we can combine two methods as

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

- **continual methods**, 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 (2D Planar LC, CEC, MEKC).
Compatibility and selectivity considerations
– microbore, liquid (mobile, carrier, electrolyte) phase

<table>
<thead>
<tr>
<th></th>
<th>HPLC</th>
<th>HPLC</th>
<th>c ITP</th>
<th>CZE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diluting or concentrating</td>
<td>Diluting or concentrating</td>
<td>Concentrating</td>
<td>Diluting</td>
<td></td>
</tr>
<tr>
<td>Reversed Phase</td>
<td>Reversed Phase</td>
<td>pK</td>
<td>pK</td>
<td></td>
</tr>
<tr>
<td>Size exclusion</td>
<td>Size exclusion</td>
<td>Effective mobility in fully charged state</td>
<td>Effective mobility in fully charged state</td>
<td></td>
</tr>
<tr>
<td>Ion Exchange</td>
<td>Ion Exchange</td>
<td>Secondary equilibria</td>
<td>Secondary equilibria</td>
<td></td>
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<tr>
<td>HILIC</td>
<td>HILIC</td>
<td>Complexation</td>
<td>Complexation</td>
<td></td>
</tr>
<tr>
<td>Hydrophobic Interaction</td>
<td>Hydrophobic Interaction</td>
<td>Inclusion</td>
<td>Inclusion</td>
<td></td>
</tr>
<tr>
<td>Affinity IMAC</td>
<td>Affinity IMAC</td>
<td>Solvation</td>
<td>Solvation</td>
<td></td>
</tr>
<tr>
<td>Normal Phase</td>
<td>Normal Phase</td>
<td>Counterion</td>
<td>Counterion</td>
<td></td>
</tr>
</tbody>
</table>

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

Róbert Góra, Pavol Rohárík, Milan Hutta

SEC, Separon HEMA 100, DMF/buffer pH 3.00

RP-HPLC C18, DMF/buffer pH 3.00

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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)

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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

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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

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

Figure 9.10  Three-dimensional representation of the data ‘volume’ of a trypic 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.
Interfacing HPLC and ITP - CZE

Orthogonal systems for humic substances characterisation?

Hutta M., Gora R. 2003

Nagyova I., Kaniansky D., 2001

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Future trends?

- Search for orthogonal systems
- 3D 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…