

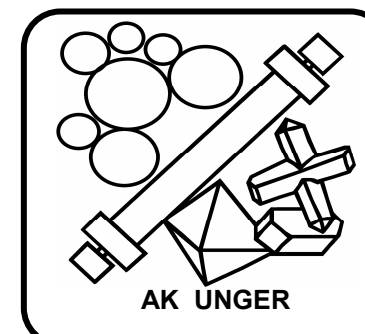
New trends in packings, stationary phases and columns in HPLC

K.K. Unger, E. Machtejevas, R. Skudas, B.A. Grimes, T. Hennesy, S. Luedtke, C. du Fresne, M. Quaglia, V. Kudirkaite

Institute of Inorganic Chemistry and Analytical Chemistry,
Johannes Gutenberg-University, 55099 Mainz, Germany



October 12, 2006, Grobbendonk ,Belgium



Outline of presentation

- 1. The current situation and the needs**
- 2. Basics of HPLC – a reminder**
Redefinition of chromatographic resolution
- 3. Advancements in Reversed Phase Chromatography –**
Matching column selectivity
- 4. The hunt for plate counts : 1 – 2 μm particle columns**
- 5. Monolithic columns: an alternative ?**
- 6. Column miniaturization: predictions and reality**
- 7. Polymer based vs. silica columns**
- 8. Conclusion and perspectives**

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The global players applying HPLC: Pharmaceutical and chemical industry

Tasks :

**Separation of low molecular weight analytes
(< 1, 000 g/mol) out of synthetic mixtures and from
biofluids**

Areas : Drug discovery, impurity profiling etc.

Needs :

**Optimum resolution, fast and rugged analysis,
hyphenation to MS, high throughput, automation,
high reproducibility**

Today's solutions :

Short reversed phase columns packed with particles smaller than 5 μm , operated isocratically or in the gradient elution mode hyphenated to mass spectrometry (off-line and on-line)

Future alternatives:

**Micro columns and fused silica capillary columns packed with 1 – 2 μm particles operated at elevated column back pressures, hyphenated to MS;
Silica monoliths as micro columns and fused capillary columns (reversed phase LC)**

The global players applying HPLC: Biotech industry, pharmaceutical industry

Tasks :

Separation of biopolymers (recombinant proteins, peptides, etc) and viruses, resp.;

Isolation and purification of targets at larger quantities;

Analysis of biofluids (proteomics, peptidomics, metabolomics);

Information on constituents at low concentration e.g. biomarkers applying MS, data bases and bioinformatics

Multidimensional LC/MS

Search for solutions

Bioprocessing:

Design of adsorbents (reversed phase, ion exchange, affinity) with high selectivity and high capacity (mass loadability)

Proteomics :

Effective removal of highly abundant constituents, high resolution by multidimensional LC (ion exchange, reversed phase, affinity) ; MS (MALDI, ESI)

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The forgotten relationship: Chromatographic resolution R_s

$$R_s = t_{Rb} - t_{Ra} / \sigma_{ta} \quad (t_{Rb} > t_{Ra})$$

σ_{ta} = standard deviation of peak a in time units

$$R_s = (r_{ba} - 1) (k_b / k_a + 1) N_a^{1/2}$$

$$r_{ba} = k_b / k_a \quad (k_b > k_a) \quad \text{Selectivity coefficient}$$

Chromatographic resolution is thus dependant on

1. Selectivity term : $(r_{ba} - 1)$
2. Retardation term : $(k_b / k_a + 1)$
3. Dispersion term : $N_a^{1/2}$

Adjustment of the selectivity coefficient

$$r_{ba} > 1.0 \quad (1.01 - 1.2)$$

The selectivity coefficient is a thermodynamic parameter characterizing the distribution equilibrium of the solutes a and b between the stationary phase and the mobile phase of the column.

Optimization of the selectivity coefficient by

- selection of the stationary phase;
- selection of the mobile phase;
- (column temperature);
- applying multidimensional LC

Method Development in POPLC™ (Phase Optimized Liquid Chromatography)

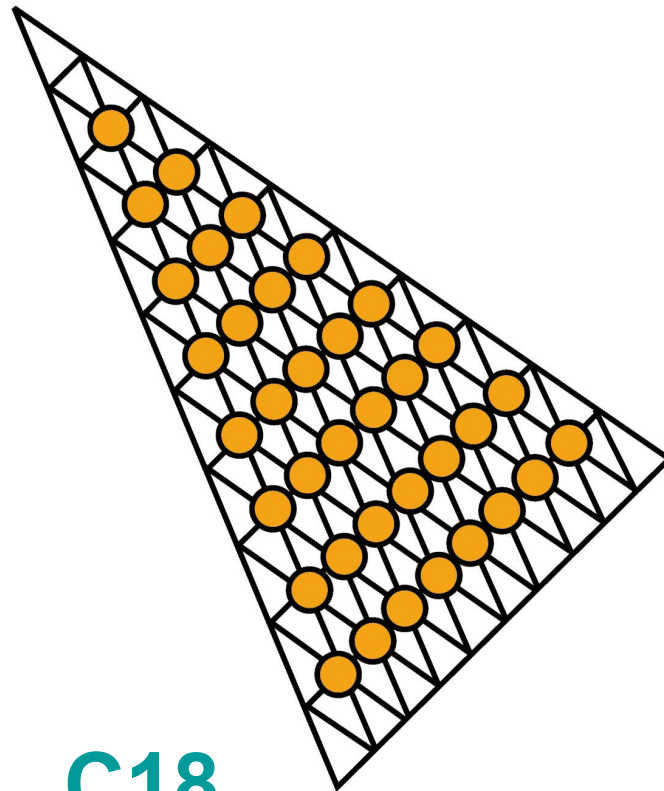
Procedure

- rough choice of mobile phase (% organic, type, pH)
- one base measurement on each of n (often 3 to 5) different stationary phases
- Determination (optimization) of the optimum stationary phase

Scheme of POPLC™

most common example to start with

Polar embedded C18

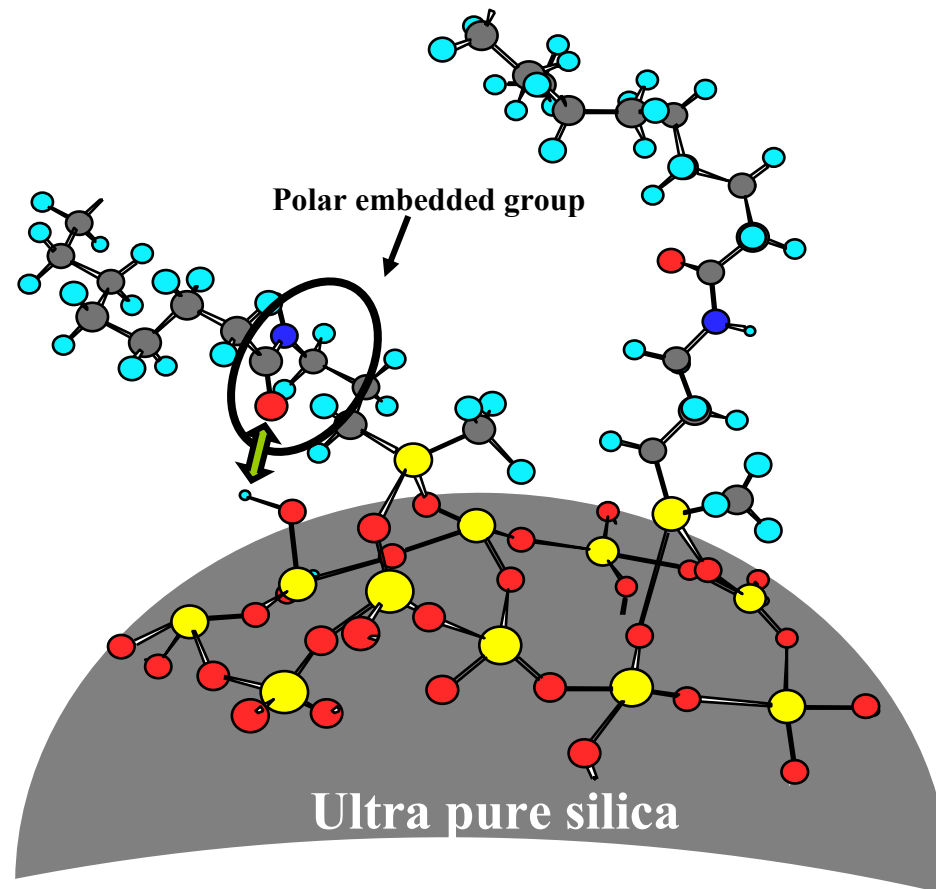


C18

Phenyl

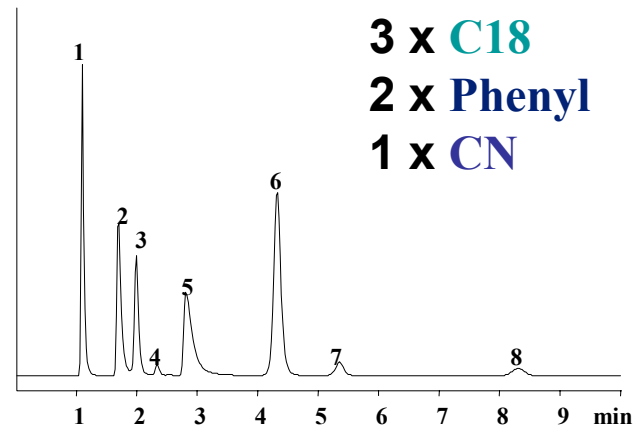
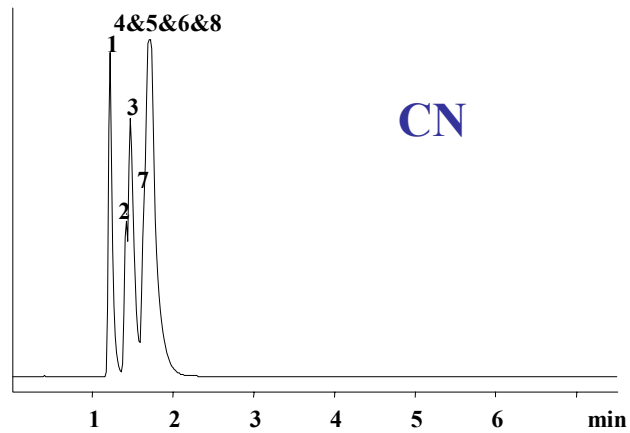
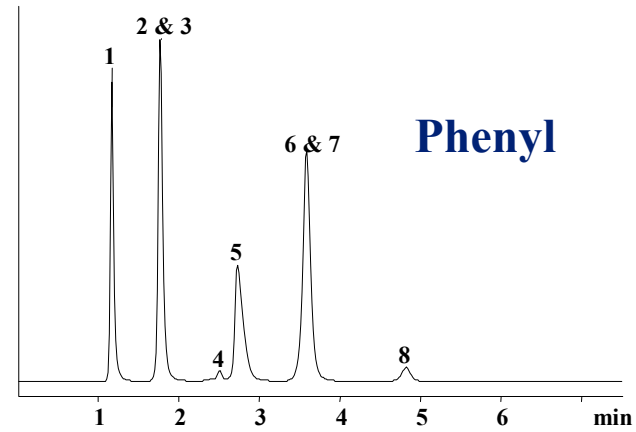
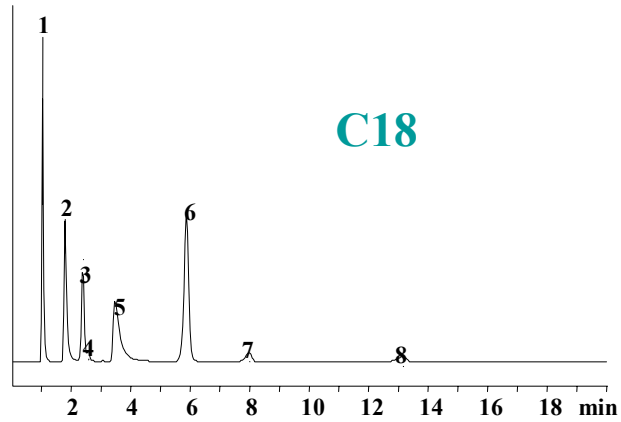
Polar Embedded Phases

Surface of ProntoSIL C18 ace-EPS



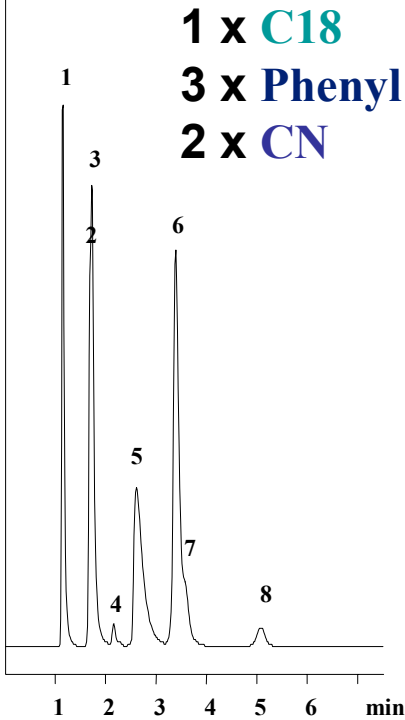
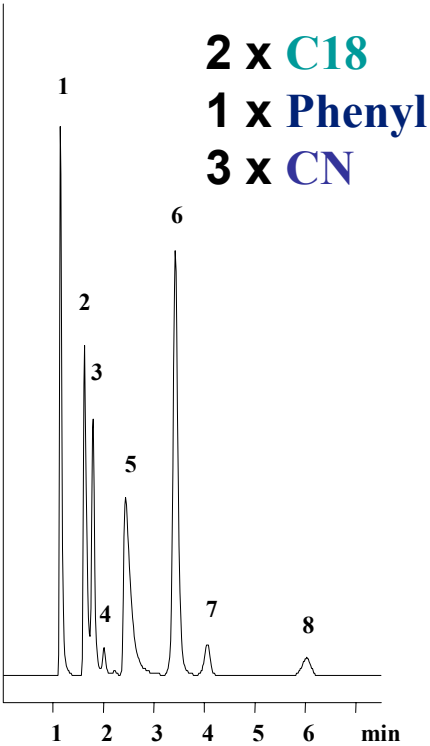
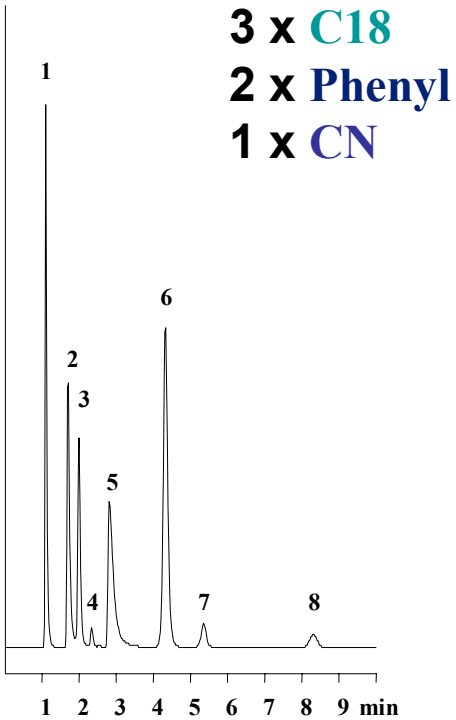
Example

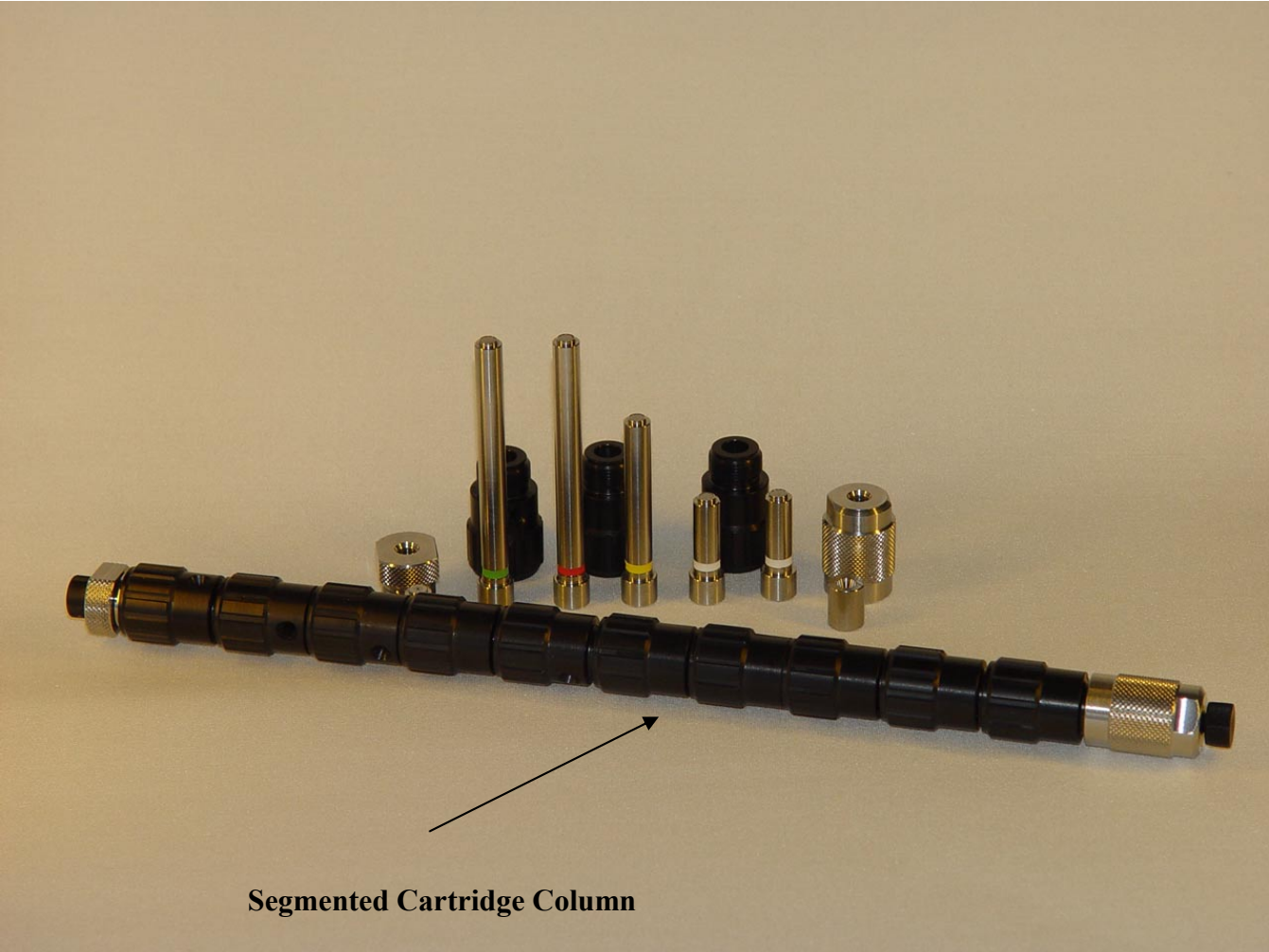
first empirical experiments



Example

Comparison of different segment compositions





Segmented Cartridge Column

Advantages of POPLC™

- **in most cases no gradient elution required, therefore**
 - ☺ **constant detector background**
 - ☺ **less requirements for the HPLC device**
 - ☺ **no re-equilibration (faster analysis)**
 - ☺ **re-usable mobile phase**
 - ☺ **detectors like RI, EC and Conductivity can be used**
- **simple method optimization using a software**
- **easy replacement of column parts**
- **Method can be used in all formats from micro to prep LC**
and can be applied to all kinds of chromatography (GC, DC,
etc.)

Source: St. Lamotte, Bischoff Chromatography, Leonberg

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Column watch by R. Majors Pittcon 2006

Take home message :

**Reversed Phase HPLC with C18 surface
functionality is still dominant;**

**Alternatives : C8, C4, polar embedded C18,
cyano, amino (NPC, RPC), C30, phenyl, fluoro
bonded**

**Zirconia, titania, polymer coated, polymeric,
hybrid stationary phases**

**Particle and column I.D. are getting smaller:
dp < 2 μm , column I.D. 1 – 2 mm and < 100 μm**

Table III: Advanced native and bonded silica columns developed by Jack and his collaborators at Agilent Technologies

Type and Assignment	Specific Structure and Surface Chemistry	Recommended Use in LC
Normal Phase columns		
Zorbax Rx-SIL	highly pure silica, low surface acidity	basic analytes
Reversed Phase columns		
Stable Bond (SB) columns (8 nm)	monofunctional silanes with diisopropyl side groups and C8, C3, Phenyl, Cyano terminating groups; no endcapping monofunctional silanes with diisobutyl side and terminating C18 groups; no endcapping	low pH separations with acidic mobile phases superior stability and reproducibility; wide window of selectivity; choice of high temperature operation (SB C18 up to 90 °C)
StableBond (SB) columns (8 nm, 30 nm)	ultrapure Zorbax Rx silica as supports with 8 nm and 30 nm pore size, resp. diisopropyl side groups and C8, C3, Cyano terminating groups; no endcapping monofunctional silanes with diisobutyl side and terminating C18 groups; no endcapping	separation of biologically active analytes e.g. peptides and proteins at low pH high temperature operation (SB C18 up to 90 °C)
Zorbax Eclipse XDB columns (eXtra Densely Bonded)	Zorbax Rx Sil as a support (8 nm pore size), dimethylalkylsilyl spacer with terminating phenyl, C 8, and C18 groups, resp.	basic compounds, stable at low to mid pH mobile phases, rapid separations pH working range between 3 and 8
Zorbax Extend-C18 (bidentate silane)	Bidentate silane with C18 groups	Increases utility of silica-based packings to high pH limits (useful in pH range of 2.0 to 11.5).
Poroshell 300SB-C18 column	solid core with a porous shell of 30 nm pore size stable bond C 18 functionality	very fast protein separations at low pH, high column efficiency

How many types of columns do we need ?

There will be no universal HPLC column for all kinds of purposes.

**Group specific columns
(acids, bases, specific pH range)**

Specialty columns for special applications

**Single client columns
(enantiomer separations)**

Measures to be taken when applying novel column types

Methodology transfer

(adjustment of eluent composition, flow-rate, column temperature adjustment , gradient conditions, etc.)

Validation process

Often neglected :

column mass loadability, particular for capillary columns in MD-LC, data sampling rate of detector at fast separations and others

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The role of particle size in LC – a brief historical review

Predictions out of fundamental studies by Huber, Halasz, Knox and others were:

Reduce the particles size of packing to make separations faster and more efficient.

The technical barriers to overcome the use of microparticular packings were :

Novel synthesis protocols

Novel sizing techniques

Appropriate column packing technologies

Fundamental and future directing papers:

I. Halasz, R. Endele and J. Asshauer, J. Chromatogr, 122
(1975) 37 – 60

Ultimate Limits in HPLC

At a column pressure of appr. 500 bar the temperature of the eluent may increase up to 35 degrees Celsius. Temperature and viscosity gradients exist in axial and radial directions inside the column. For routine work the particle size should be $5 \mu\text{m} < d_p < 3 \mu\text{m}$. The minimum particle size in LC is appr. between 1 and 3 μm .

Main message : Maximum pressure of LC equipment should be appr. 400 bar.

Is there any need to apply packings with a mean particle diameter of $d_p \ll 5 \mu\text{m}$ in HPLC ?

benefits

- high column efficiency
- short analysis time
- high detection sensitivity

precautions,
limitations

- column design (length, bore, frits etc.)
- instrument design to eliminate extra column effects
- frictional heating and temperature effects
- maximum pressure drop

H. Giesche, K.K.Unger, U. Esser, B. Eray and U. Trüding *J. Chromatogr.* **465** (1989) 39 – 57

Packing technology, column bed structure and chromatographic performance of 1 – 2 μm nonporous silicas in HPLC

Aggregate formation studied by different techniques, slurry packed columns of 53 x 4.6 mm packed with 2.1 μm C18 bonded nonporous silicas, generating plate heights between 5 and 8 μm at linear eluent velocities between 2 and 6 mm/s, flow-rate at 2.5 ml/min generate a column back pressure of 500 bar, to keep extra-column effects to a minimum, the injection volume should be appr. 0.6 μl , the volume of the detector cell about 0.3 μl and the time constant of the detector < 60 ms, such columns enable separations of low molecular weight analytes of less than 60 s.

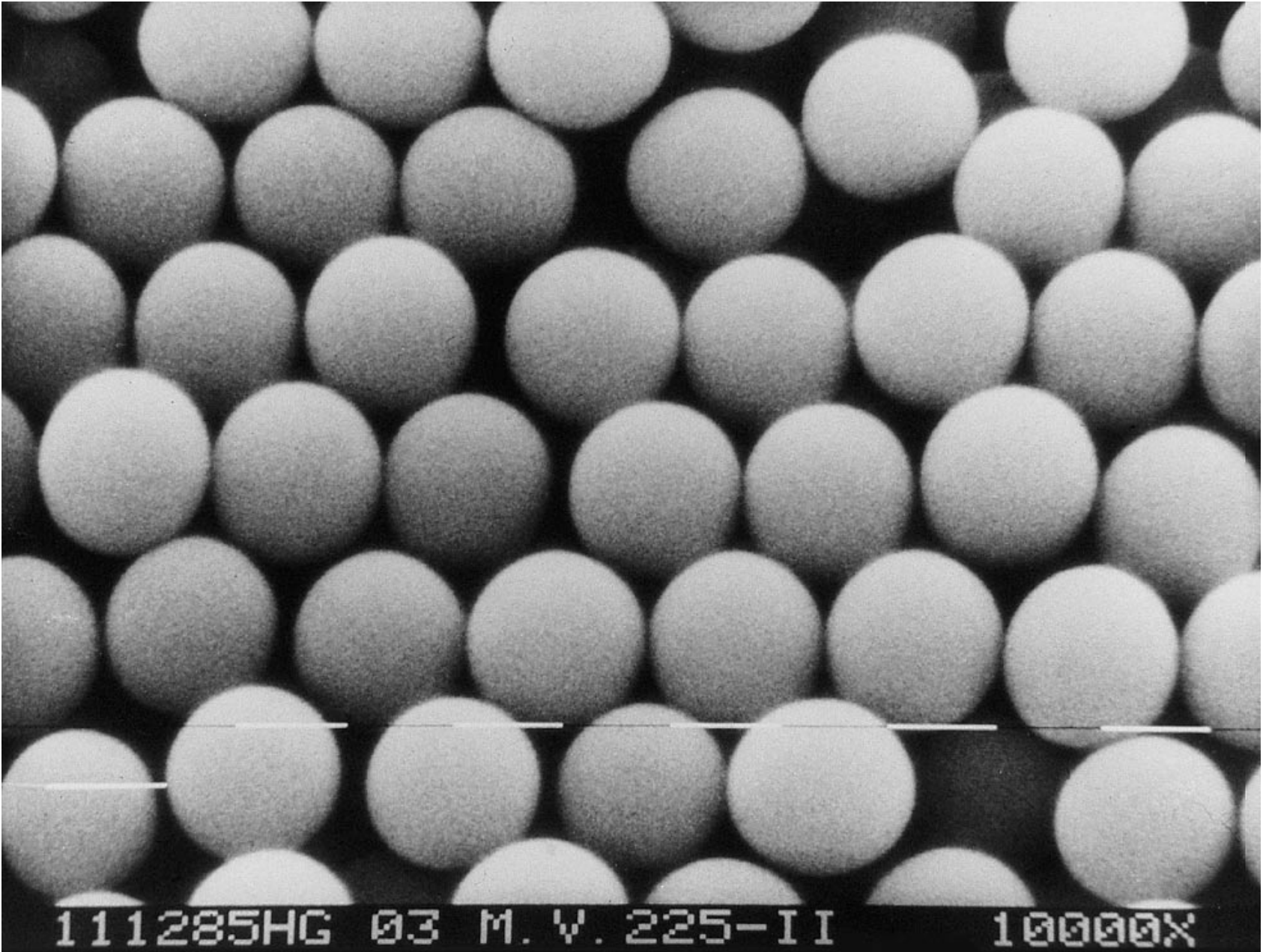
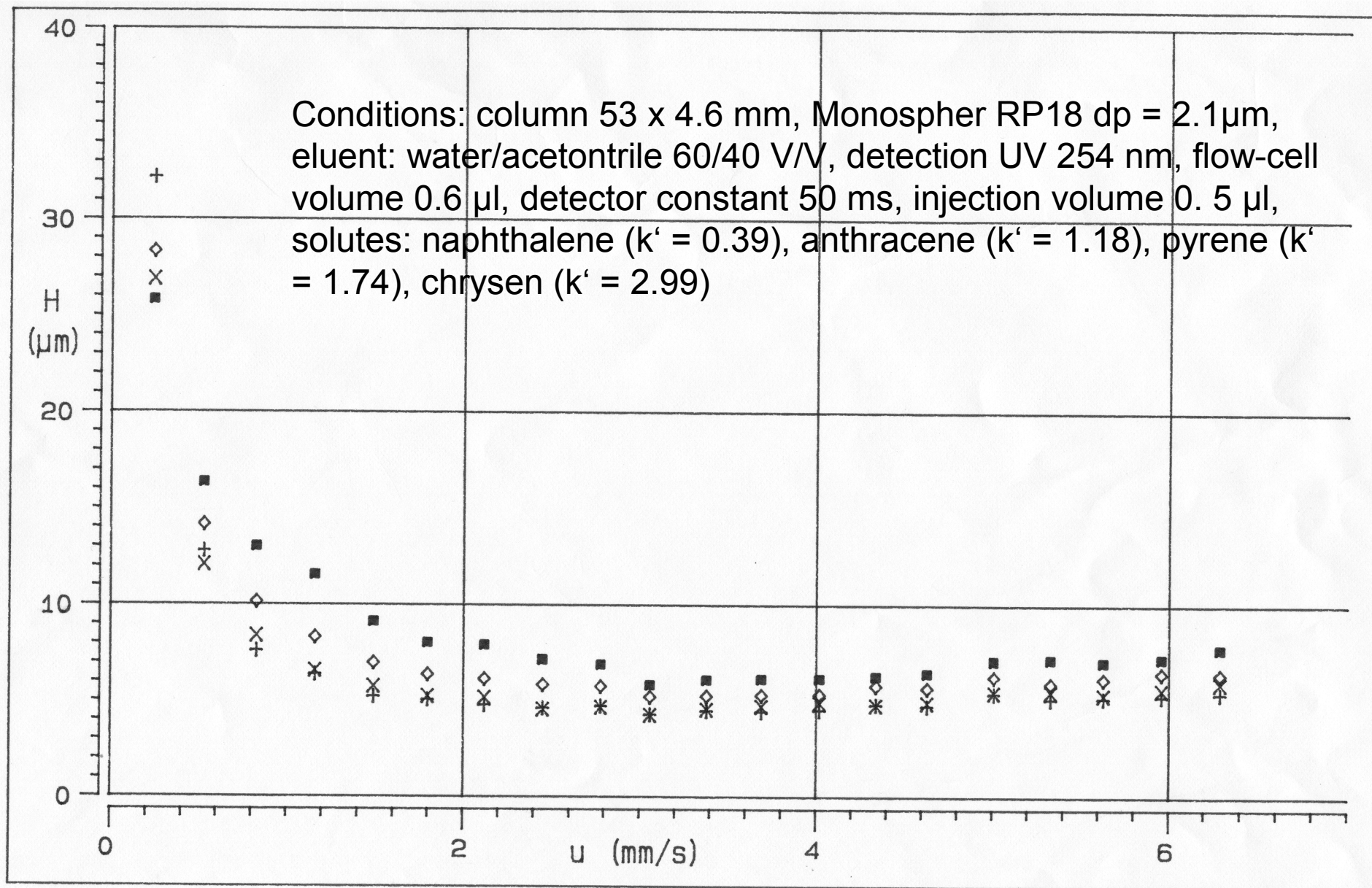


Plate height – linear velocity plots on a 2 μm nonporous RP 18 silica column



Column back pressure ΔP

$$\Delta P = \frac{\eta \cdot u \cdot L \cdot \Phi}{d_p^2}$$

$$\eta = 1 \text{ mN}\cdot\text{s}/\text{m}^2$$

$$u = 5 \text{ mm}/\text{s}$$

$$L = 53 \text{ mm}$$

$$\Phi = 620 (\varepsilon_t = 0.35)$$

$$d_p = 2.1 \mu\text{m}$$

$$\Delta P = 37.3 \text{ MPa} = 373 \text{ bar} = 5600 \text{ psi}$$

Conclusions

porous $1\mu\text{m}$
beads

- problems still exist in preparing materials being free of fines of $d_p < 0.1\mu\text{m}$
- particle porosity has to be adjusted with regard to the high packing pressure
- columns of $< 50\text{mm}$ length have to be employed to avoid high back pressures
- achievement of optimum performance is only possible by appropriate design of the instrument (injection, detection, tubings etc.)

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Monolithic columns :

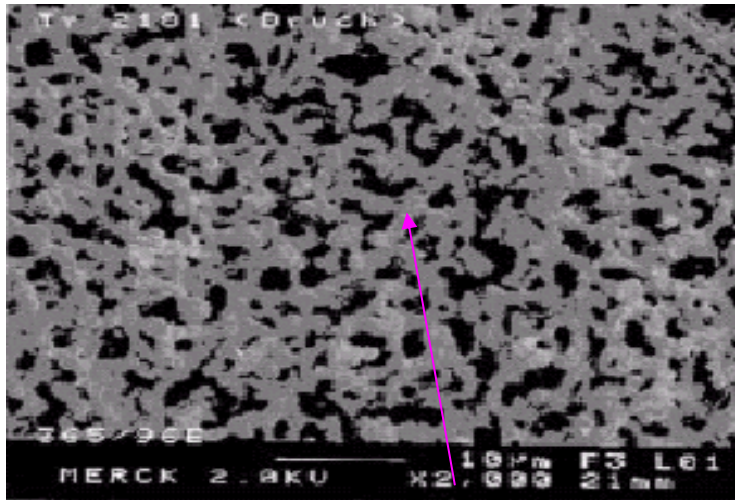
The basic concept

Advantages : no frits needed, substantial gain in robustness, adjustment and control of mass transfer kinetics of solutes at a continuous bed column, improvement leads to shorter analysis time, higher mass loadability due to better utility of ligands, higher peak capacity.

Specific features for monolithic silica columns as compared to polymer based:

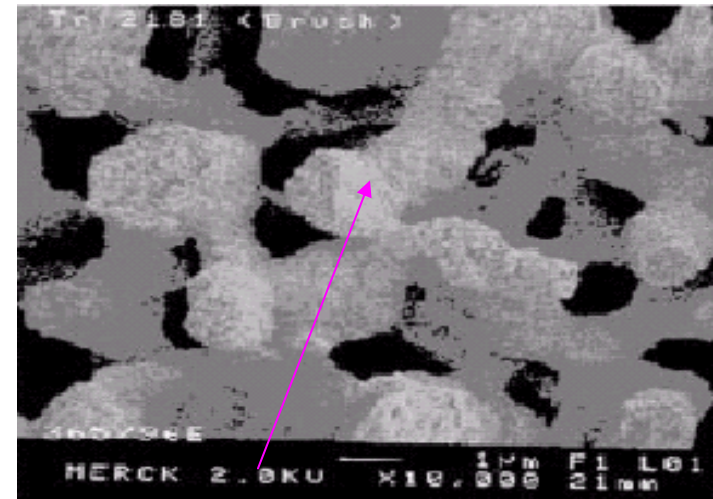
Independent control of mesopore size and macropore size due to the formation processes, independent control of macroporosity and mesoporosity, optimum design with respect to high pore connectivity.

Pore structure and morphology of monolithic silica capillaries



Macropores
(through pores)

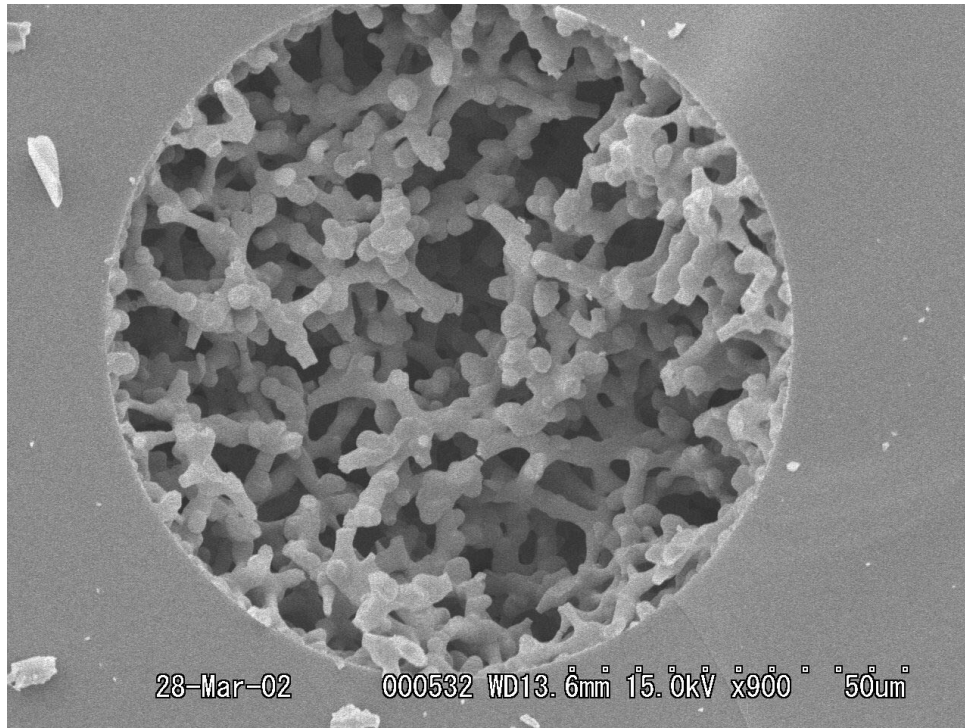
Permeability, mass
transfer resistance



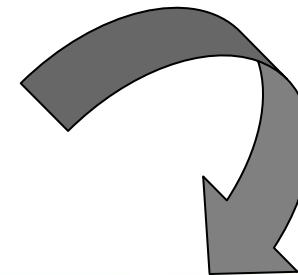
Mesopores (located
in the skeleton)

Retention and
mass loadability

Monolithic silica capillaries

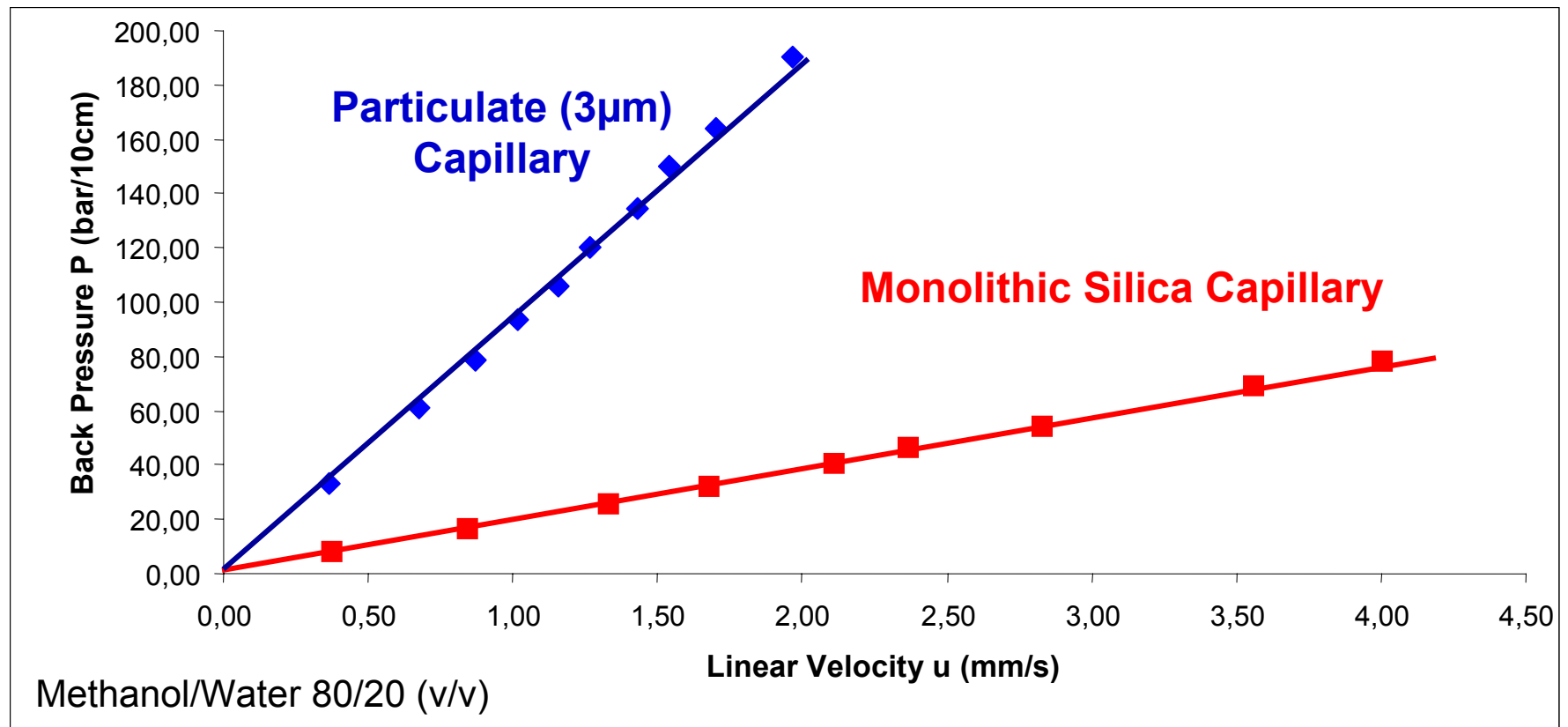


Capillary 100 μm I.D.



Column Back Pressure

P/u curve of monolithic and particulate Capillaries



Comparison of essential features of monolithic capillary columns in LC

	Advantages	Disadvantages
Monolithic silica columns	Optimum design of pore structure and functionality	pH limitation
Monolithic polymeric columns	Functionality can be implemented in the manufacturing process	Pore structure not yet optimized, high back pressure

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Options in LC technology

	column I.D / mm	flow-rate / $\mu\text{l}/\text{min}$
Meso	4.6	1, 000
	4.0	800
	2.0	200
	1.0	50
Micro	0.3	5
	0.1	0.5
Nano	0.05	0.1
	0.02	0.04

Column dimensions are dictated by the analytical needs !

High pressure LC: the pioneering work by Jorgensen

**J. MacNair, K.D. Patel and J.W. Jorgensen, Anal. Chem. 71
(1999) 700 – 708**

**Ultrahigh-Pressure Reversed Phase Capillary LC:
Isocratic and gradient elution using columns packed with 1.0
 μm non-porous silica particles**

Fused silica capillaries ($L = 25 - 50$ cm, I.D. = $33 \mu\text{m}$) were packed with $1.0 \mu\text{m}$ non-porous C18 modified particles and operated at 5 , 000 bar.

The UPLC System of Waters

Separation Science Redefined ?

The UPLC system includes the following elements:

- 1.7 μm C18 bonded porous silicas**
- packed in 2.1 mm I.D. microbore columns of L = 15 cm, generating a column back pressure of 15,000 psi at optimum flow rate**
- coupled to Orthogonal Quadrupol TOF-MS(MS)**

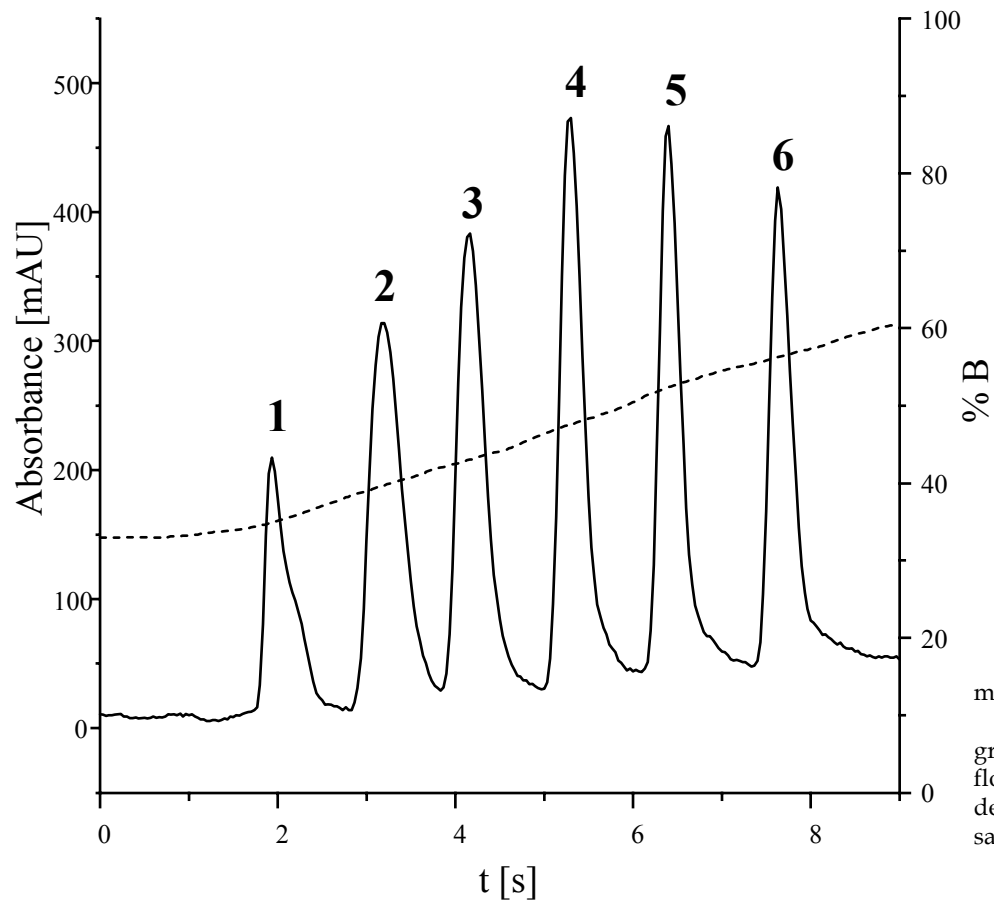
Results :

sensitivity increase 2 – 3 times higher than in conventional LC, high throughput

Ultra fast HPLC Separation

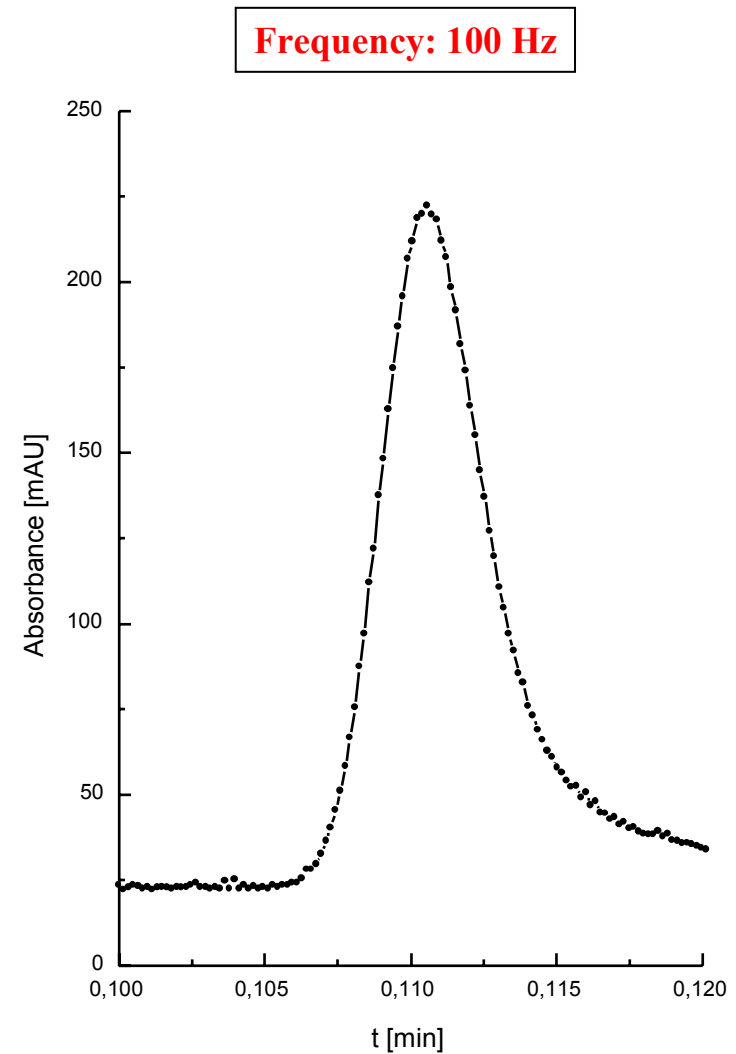
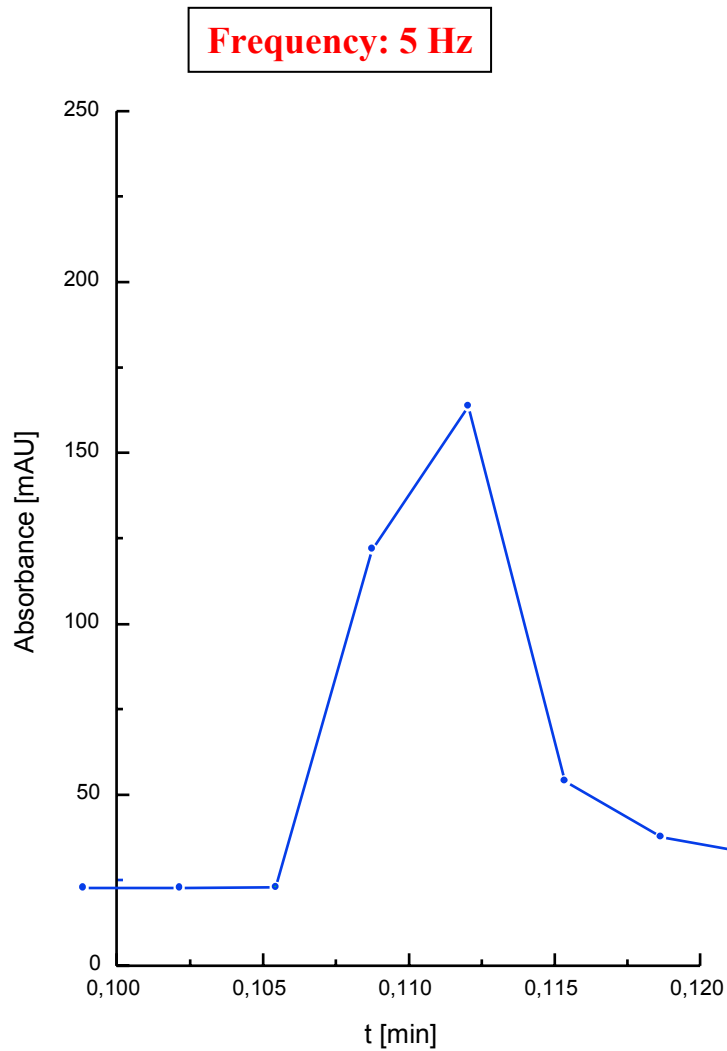
6 Compounds in 9 Seconds

MICRA NPS ODS1 1.5 μm
(14 x 4.6 mm)



Critical Parameter

Data Acquisition Rate



Solution

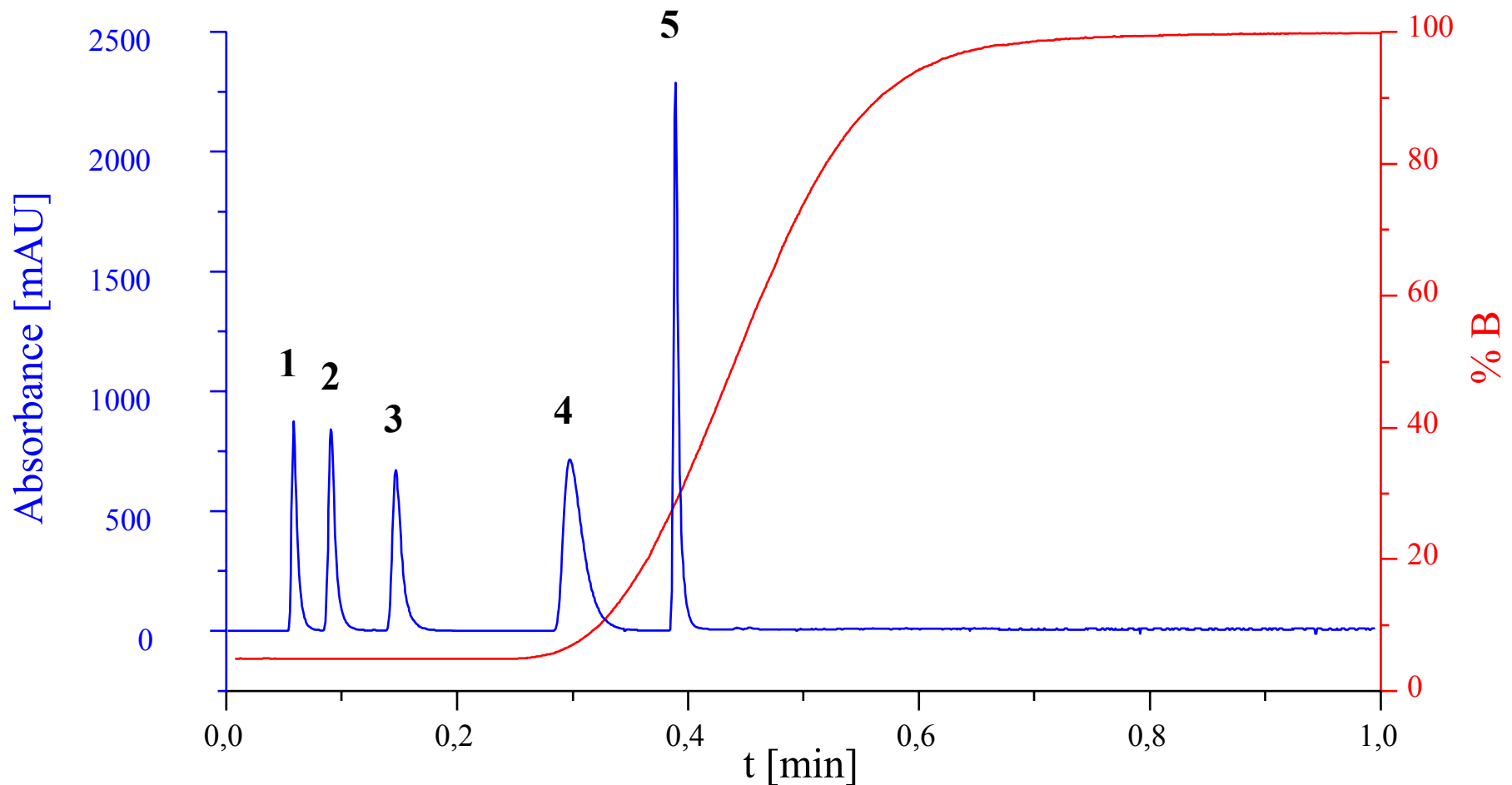
“Extra Column Effects”

- ⇒ good connections are extremely important!
- ⇒ Keep capillary lengths and I.D.s as short as possible
- ⇒ If available use a micro detector cell
- ⇒ Inject as less as possible

Critical Parameter

“Dwell Volume”

(Volume until the gradient reaches the column head)

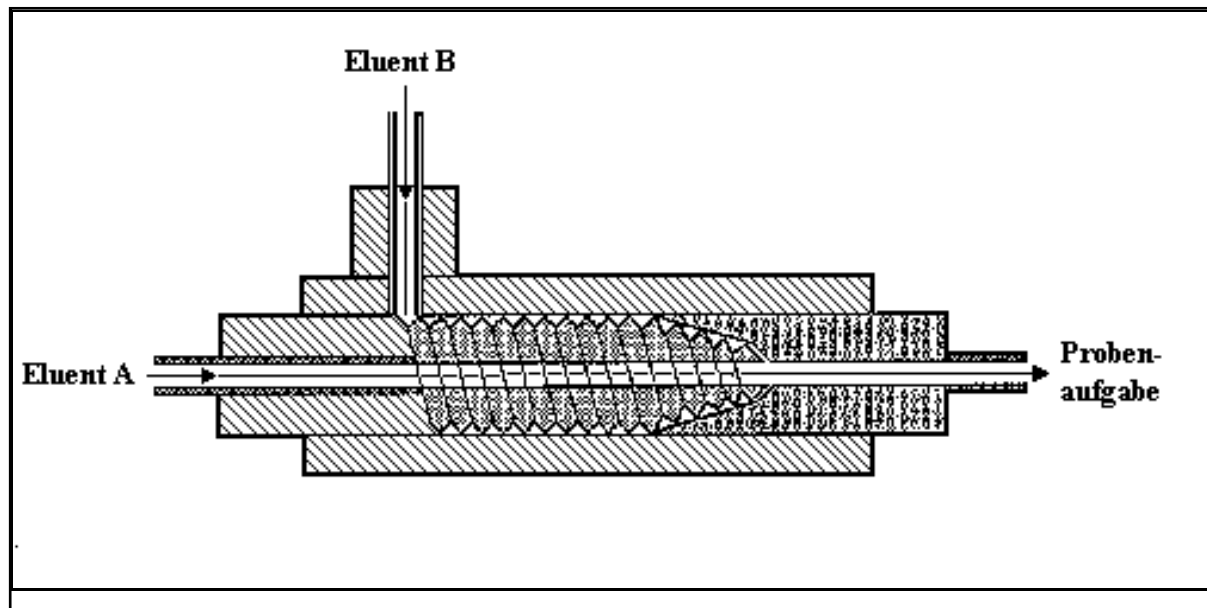


Solution

“Dwell Volume”

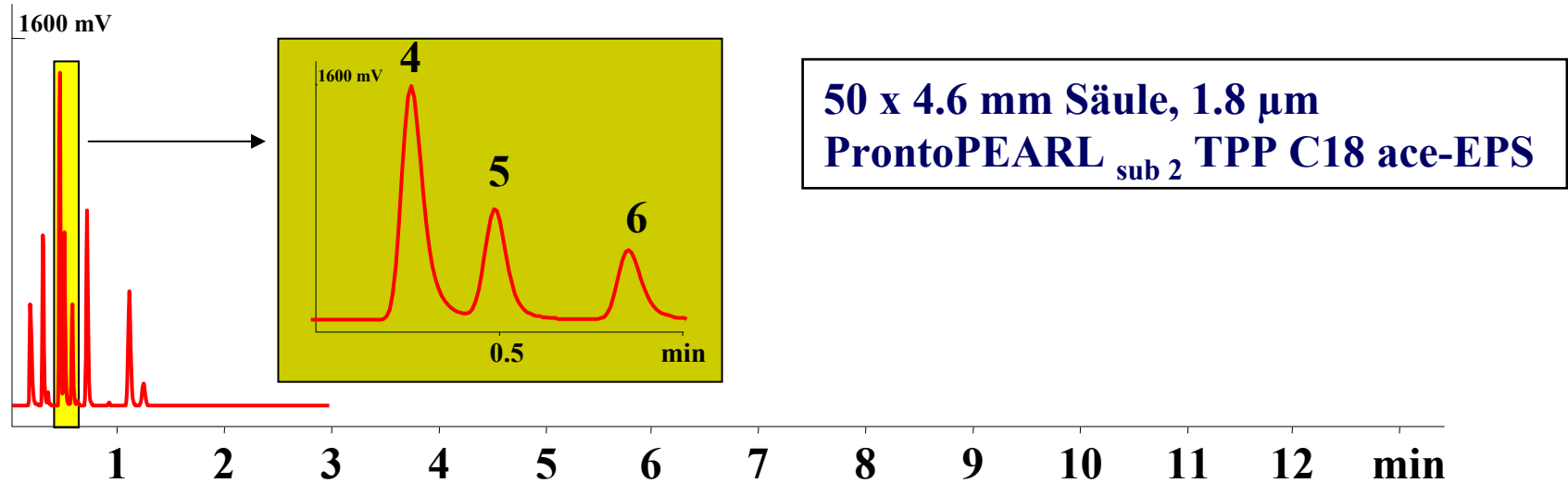
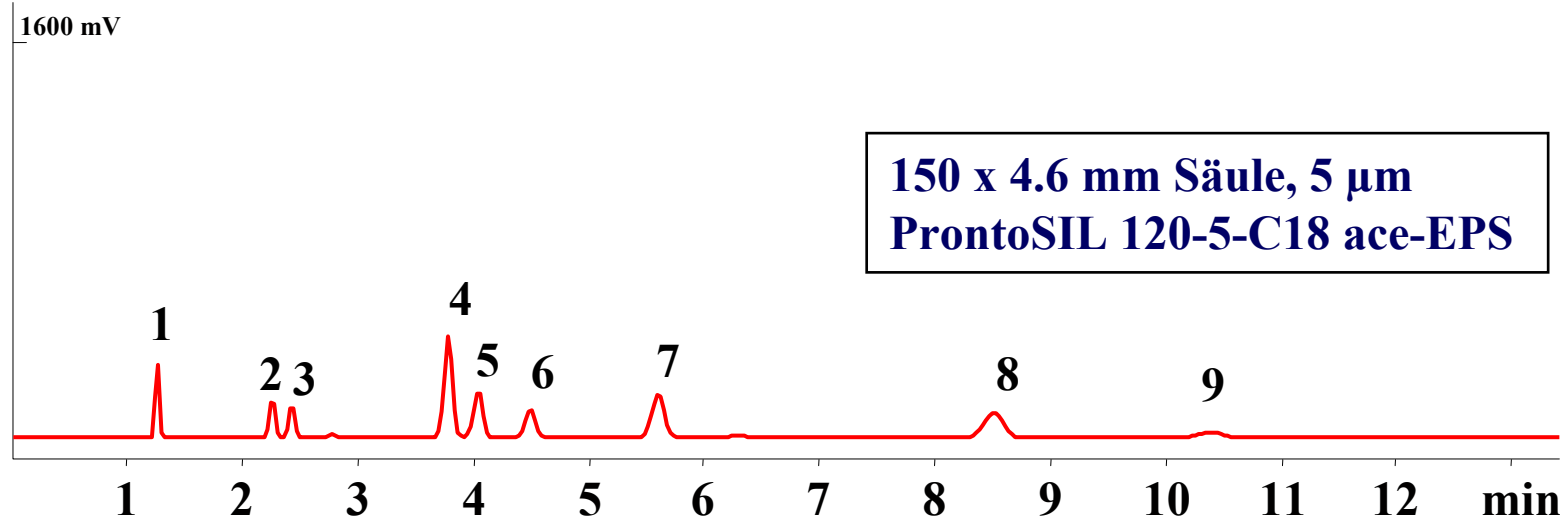
⇒ use a high pressure gradient system

⇒ use a micro mixing chamber if possible (volume max. 500 μl)



Fast HPLC

Comparison with conventional 5 μm Material



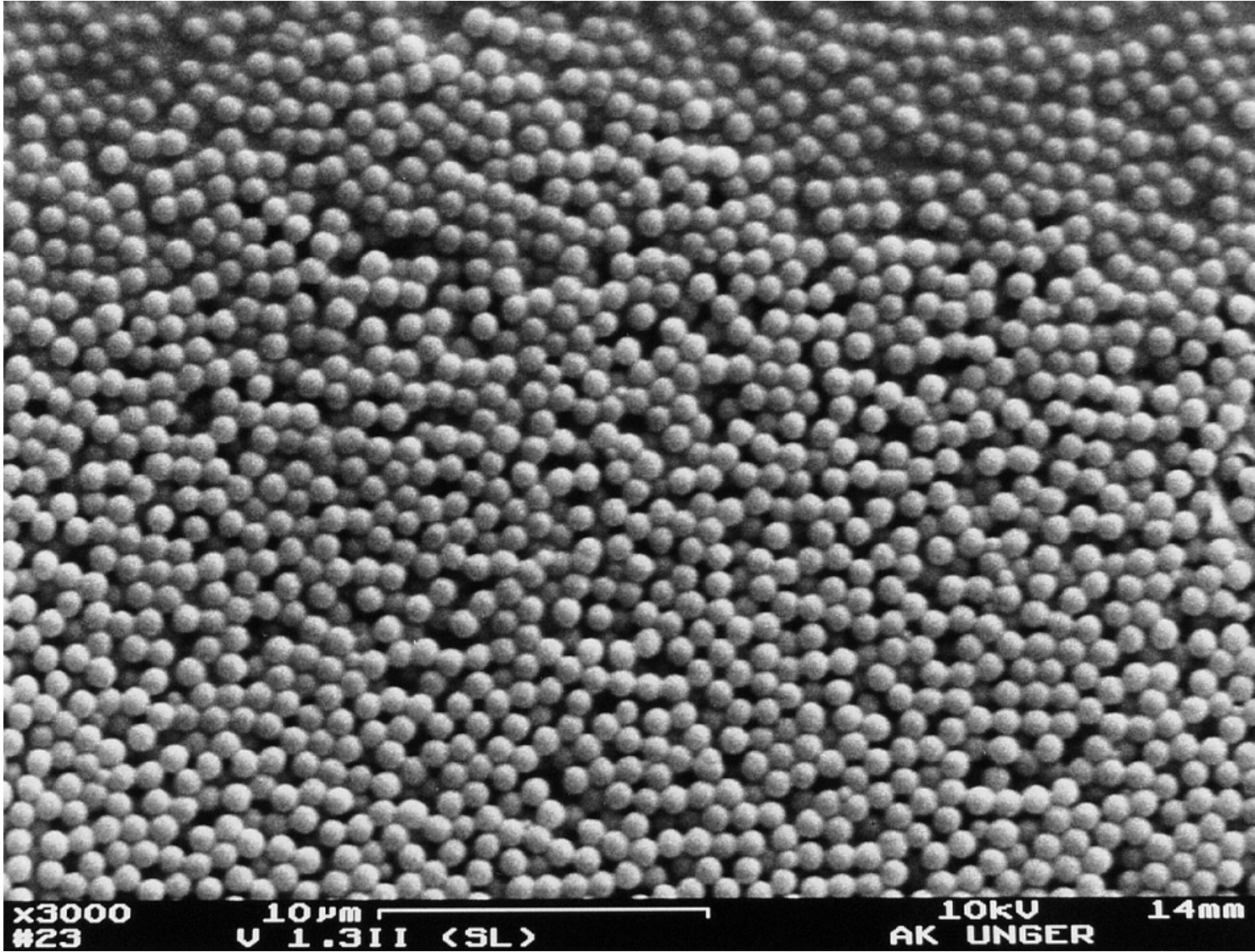
CEC – a promising separation technique for submicron size silica particles ?

Here is the answer :

**S. Luedtke, Th. Adam, N.von Doehren and K.K. Unger
J. Chromatogr. A 887 (200), 339 – 346**

Towards the ultimate minimum particle diameter of silica packings in capillary electrochromatography (CEC)

Porous silica beads with an average particle diameter between 0.2 and 3 μm , C8 surface modified were applied as packings in 100 μm I.D. fused silica capillaries in CEC, plate heights equivalent to 1 – 2 dp were generated.



x3000
#23

10 μm
V 1.311 (SL)

10kV
AK UNGER

14mm

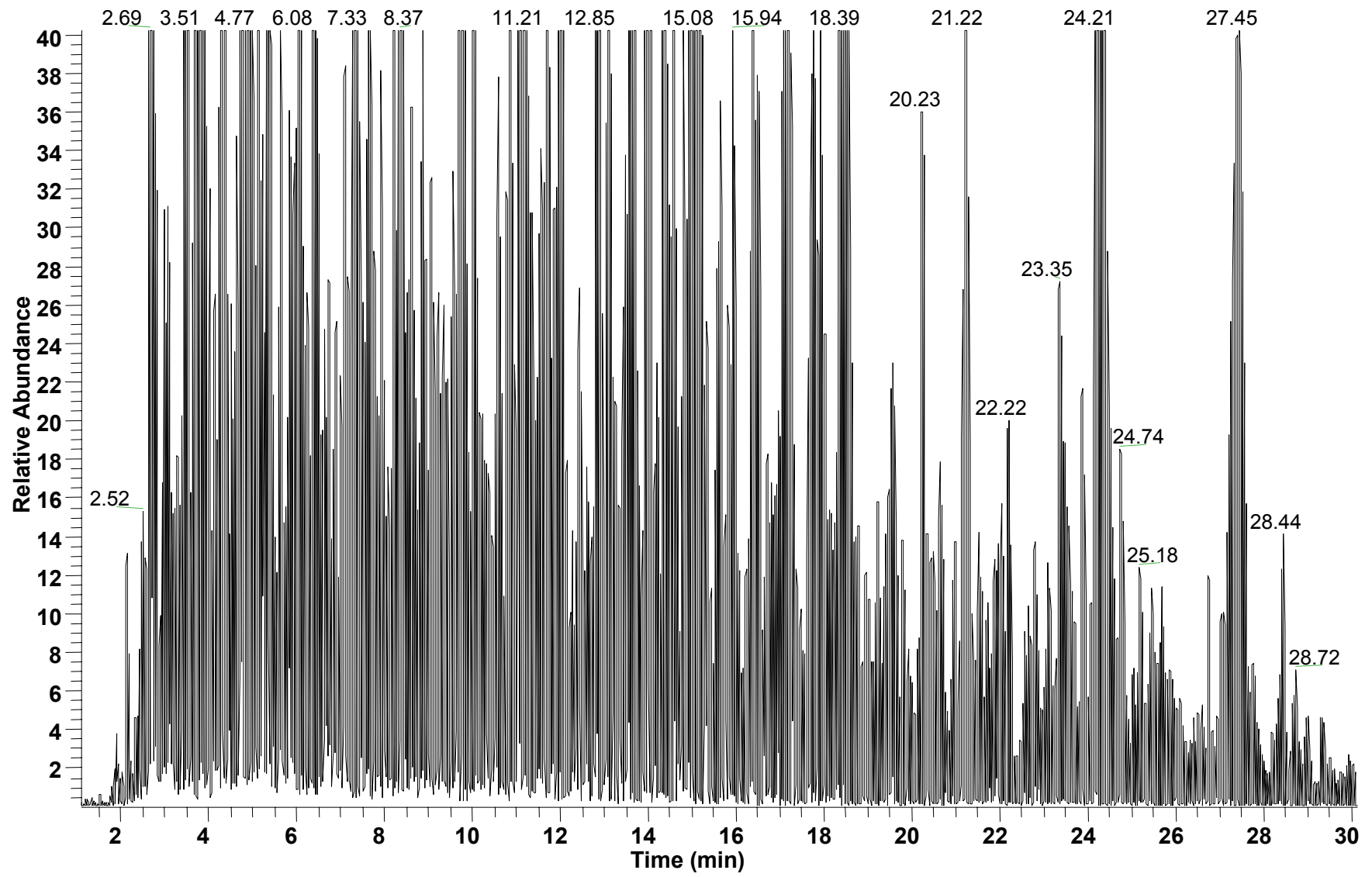
Last breaking news articles

Y. Shen , R.D. Smith, K.K Unger, D. Kumar and D. Lubda

Anal. Chem 77 (2005) 6692 - 6701

Ultrahigh Throughput proteomics using RPLC-ESI-MS/MS

Submicron C18 bonded silica particles were employed in 50 μm I.D. Fused silica capillaries in gradient elution coupled to ESI-MS/MS generating 4,000 (1, 800) peptides which enabled to identify 1,000 (550) proteins to be identified in 50 (20) minutes. Very fast RPLC separations are found to be limited by the speed of the MS/MS analysis.



Conclusion & Perspectives

There will be a continuous trend to 1 – 2 μm size packed microbore and capillary columns to generate highly efficient columns, to gain fast separations and to achieve high sensitivity when coupled to MS.

However, there are also competitive solutions by employing monolithic fused silica capillaries and chip-type of systems. Modeling of column design and engineering of systems will substantially impact the future development in LC.

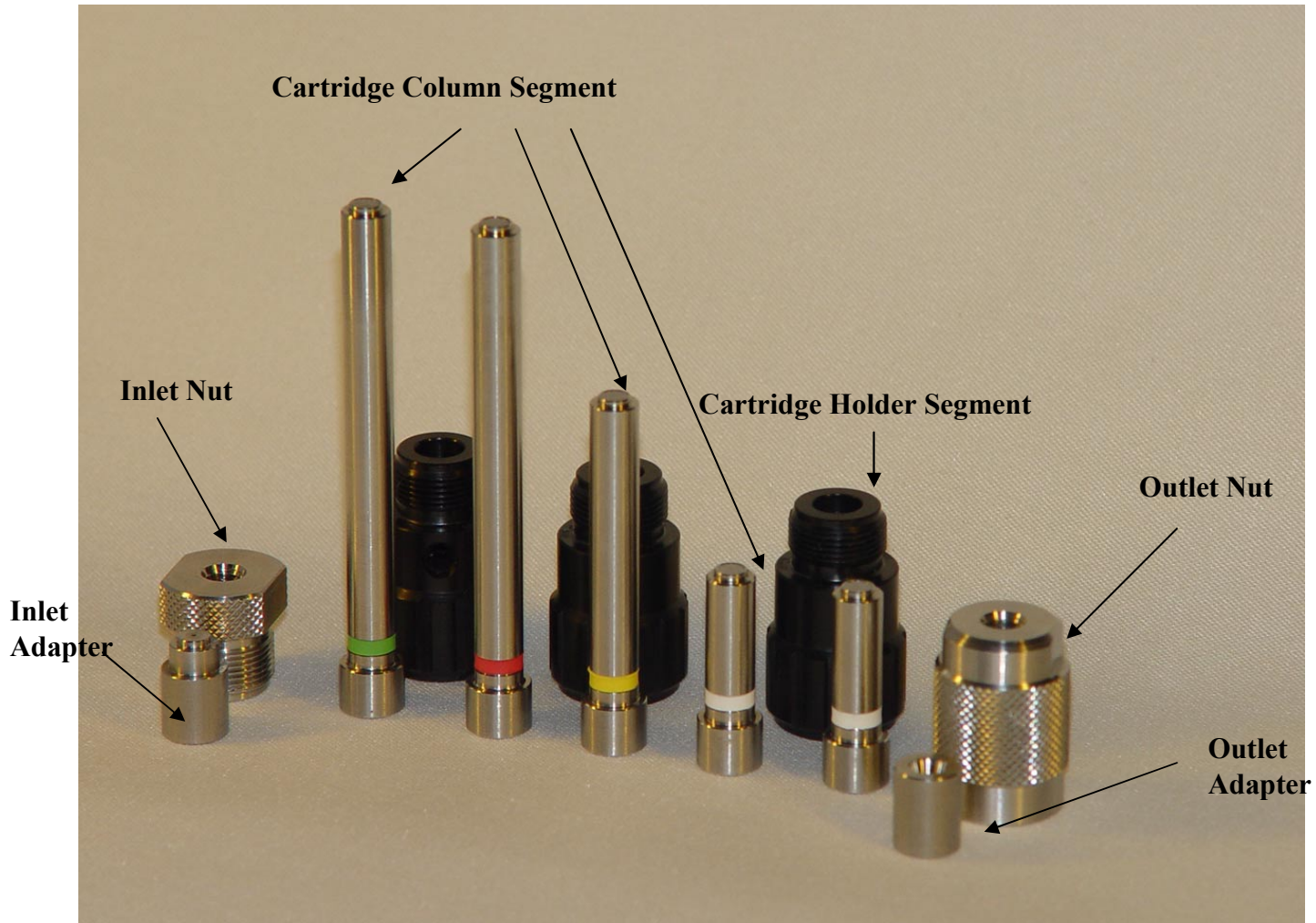
Thanks to the team



Available Cartridges



Dimensions: ID 3.0 mm
length 10, 20, 40, 60, 80 mm



State-of-art in materials and column design

Many liquid phase based separation systems still employ classical types of adsorbents .There is a lack of search for novel materials

Reason Nr. 1:

Porous adsorbents are highly complex in structure and function as compared to two dimensional systems (chips).

Reason Nr. 2:

Modeling and simulation of materials and separation processes are still at their infancy.Results of modeling need verification by experimental results.

Reason Nr. 3:

Design and development of adsorbents and columns is directed by applications (analytical, preparative, process, type of analytes and isolates etc.)

Reason Nr. 4

Material design and development is highly interdisciplinary and requires integrated research with long term planning. Today quick solutions are demanded.

Key to successful solutions

**Fundamental understanding of the basics of
separation processes**

**(hydrodynamics, mass transfer kinetics, phase
equilibria, solute-surface interactions)**

which will lead to designed adsorbents with superior
properties in terms of
particle shape and morphology, monolithic (continuous)
beds, column configuration, pore structural parameters,
surface chemistry

accompanied by modeling and simulation;
evaluation of reliable test methods and characterization
tools

Thermal effects

Viscous heat dissipation can cause a radial thermal gradient that results in peak tailing.

The maximum temperature rise along the axis of the column depends on the chromatographic parameters as

$$\Delta T = \psi \left(\frac{dc^2 \cdot v}{dp^4} \right)$$

where ψ is a constant, dc the column bore, dp the particle diameter of packing and v the reduced linear velocity of the eluent.

Miniaturization in HPLC - a hot subject over several decades

Predictions:

**G.Guiochon, Optimization in Liquid Chromatography in HPLC
Advances and Perspectives, Cs. Horvath (editor), Vol.2
Academic Press, New York, 1980, pp 1 – 54**

Experimental findings:

**H. Giesche, K.K. Unger, U. Esser, B. Eray and U. Trüdinger,
J. Chromatogr., 465, 39 – 57 (1989)**

**Presented at HPLC'88, June 19 – 24, 1988, Washington, DC,
USA**

Synthesis scheme of silica monoliths according to Nakanishi

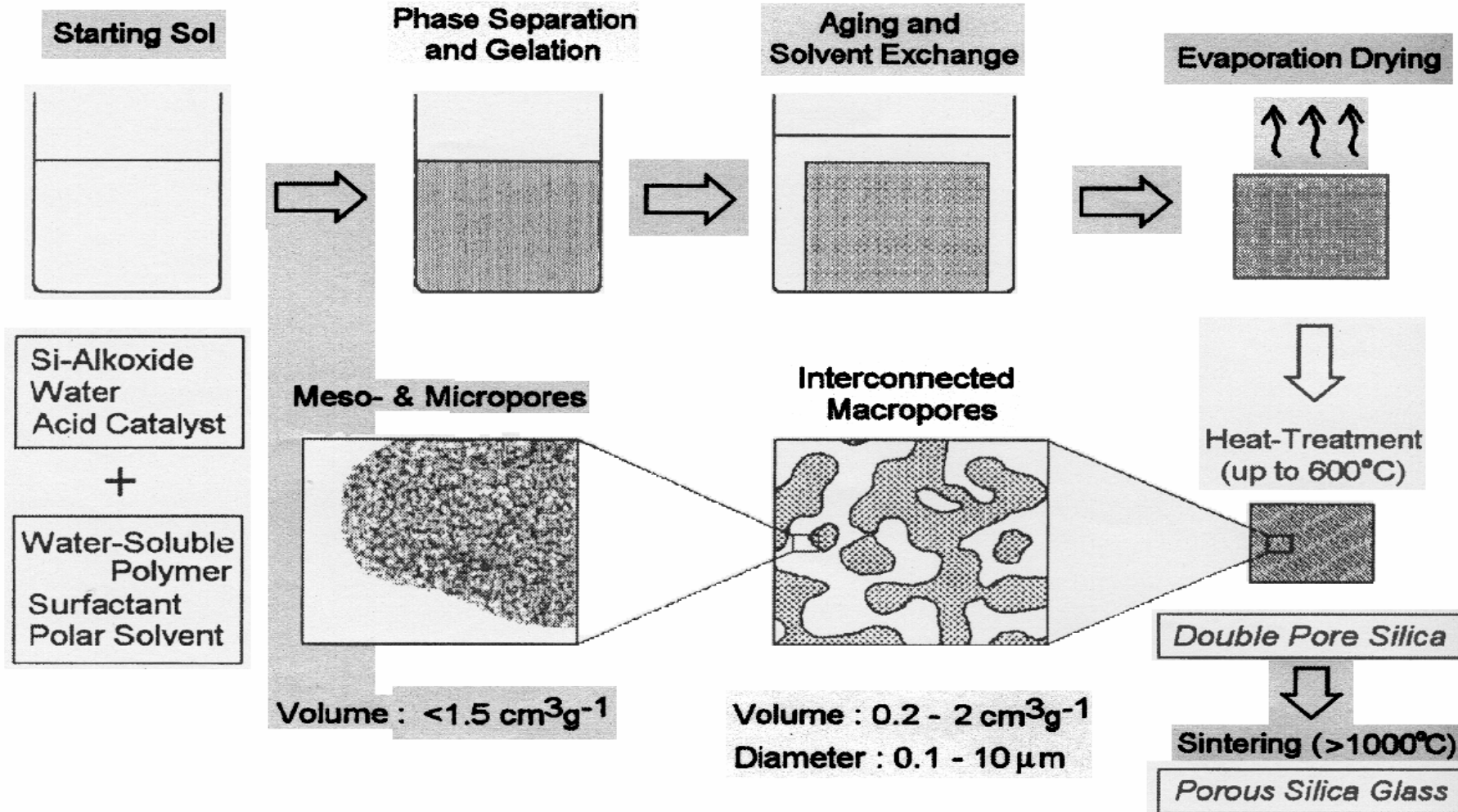


Plate height vs. linear velocity plot of a monolithic silica capillary of 100 μm I.D.

