Teil 1 - Chromatographie

Grundlagen und Anwendung moderner Trennverfahren

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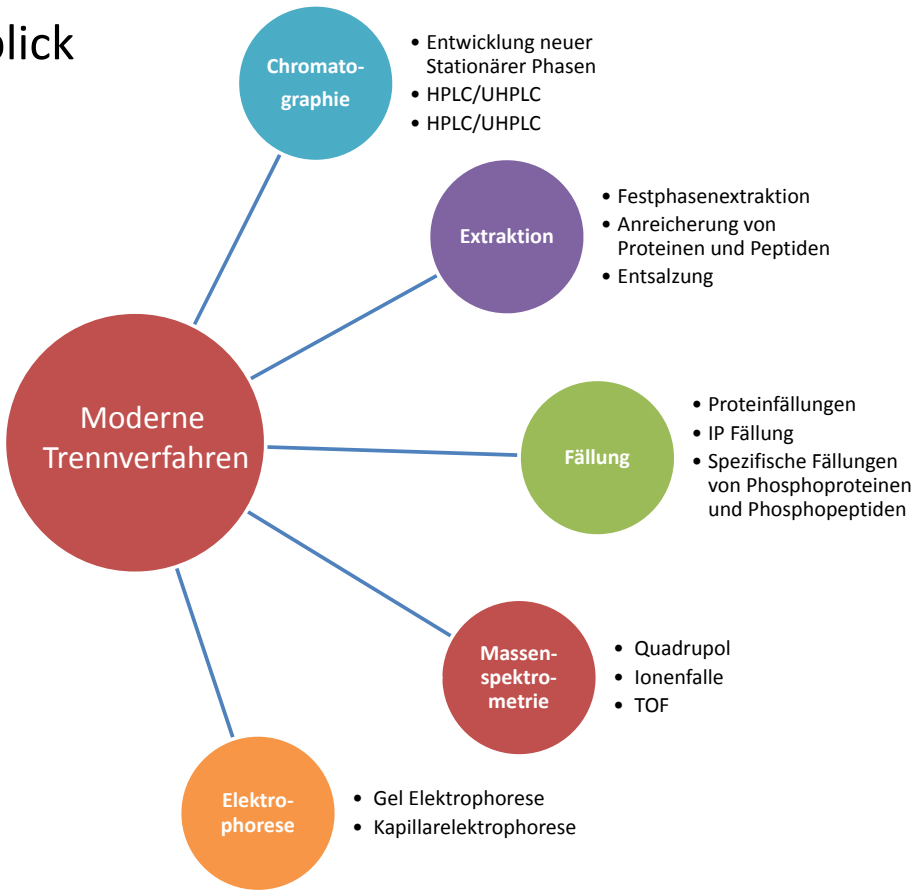
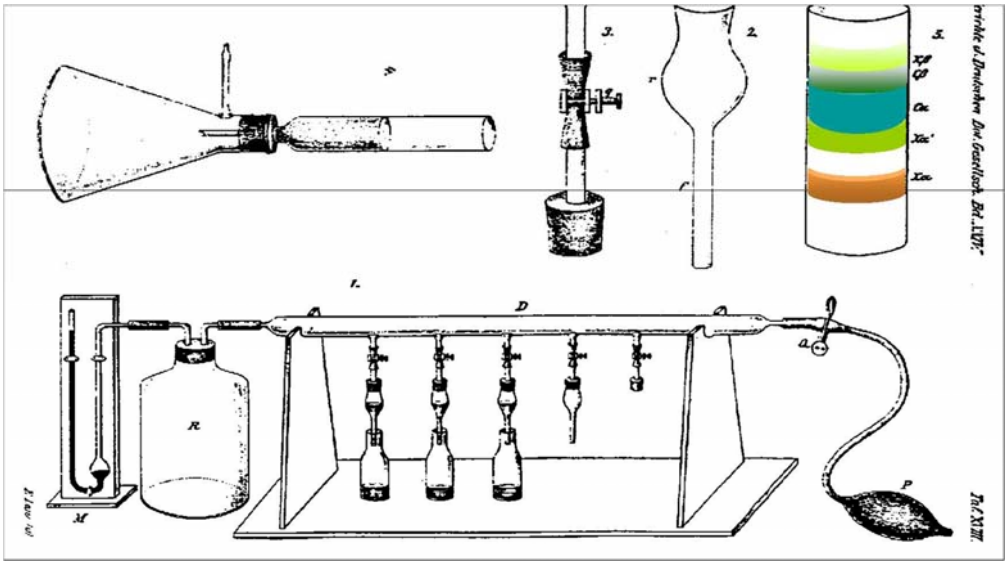
Überblick



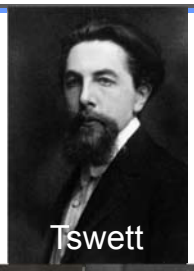
Tswett's Chromatographic System

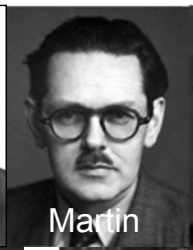
1903

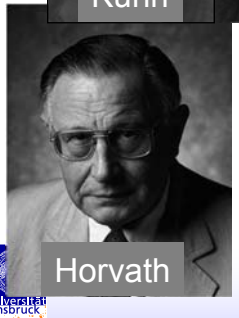


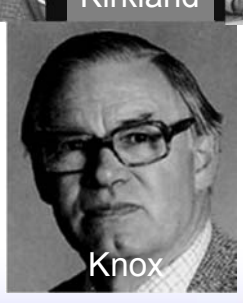
Chromatography goes a long way back

1903 Tswett
1930 Kuhn
1940 Martin
1967 Horvath
Huber
Halász
Scott









...and has many faces

First Publications found with SciFinder for High Pressure Liquid Chromatography HPLC

Horvath, Csaba G.; Lipsky, Seymour R. Column design in high - pressure liquid chromatography**.** *Journal of Chromatographic Science* **(1969),** 7(2), 109-16.

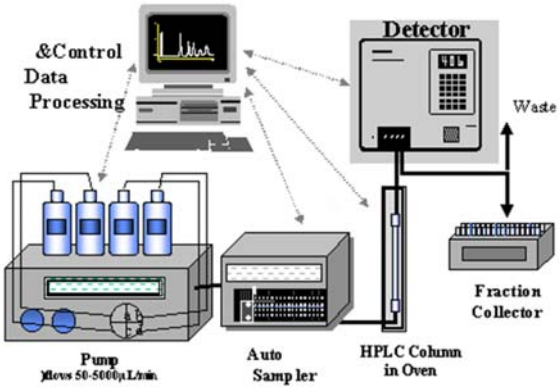
Scott C D; Johnson W F; Walker V E. A sample injection valve for use in high - pressure liquid chromatography. *Analytical biochemistry* **(1969),** 32(1), 182-4.

Felton, Herman. Performance of components of a high - pressure liquid chromatography system. *Journal of Chromatographic Science* **(1969),** 7(1), 13-16.

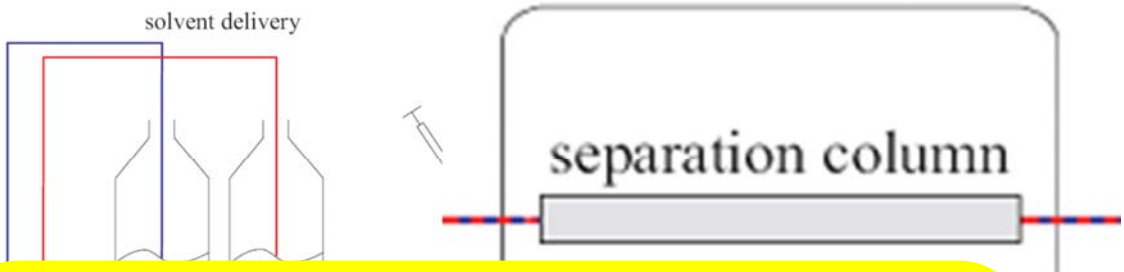
Different Aspects of Chromatography

Chromatography - Yesterday

Chromatography - Today



HPLC-Instrumentation

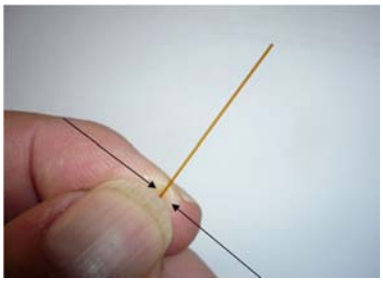


Stationary Phase: One of the two phases forming a chromatographic system. It may be a solid, a gel or a liquid. If a liquid, it may be distributed on a solid. This solid may or may not contribute to the separation process. The liquid may also be chemically bonded to the solid (bonded phase) or immobilized onto it (immobilized phase). The expression chromatographic bed or sorbent may be used as a general term to denote any of the different forms in which the stationary phase is used. *PAC 1993,65, 819 (Nomenclature for chromatography (IUPAC Recommendations 1993)*

Stationary Phases – Formats – Strategies

HPLC - column

µ-LC - capillary



Chip - LC

Disc - technology

Column Sizes

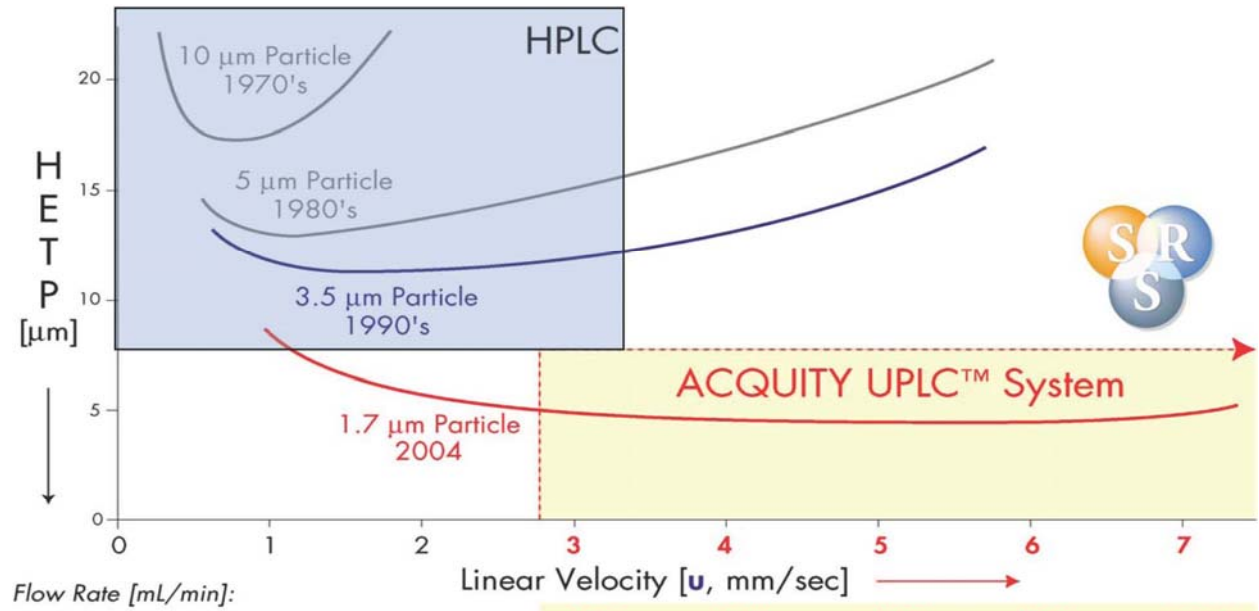
Description	Dimensions	Approx. Flow rate (velocity 1–10 mm/s)
Open tubular liq chromotgraphy	< 25 µm i.d.	< 25 nL/min25 nL/min
Nanobore column HPLC	25 µm < i.d. < 100 µm	25–4000 nL/min
Capillary column HPLC	100 µm < i.d. < 1 mm	0.4–200 µL/min
Microbore column HPLC	1 mm < i.d. < 2.1 mm	50–1000 µL/min
Narrow(small)-bore column HPLC	2.1 mm < i.d. < 4 mm	0.3–3.0 mL/min
Normal-bore column HPLC	4 mm < i.d. < 5 mm	1.0–10.0 mL/min
Semipreparative column HPLC	5 mm < i.d. < 10 mm	5.0–40 mL/min

Design changed

Waters ALC 1976

Waters ACQUITY UPLC 2004

Van Deemter



Flow Rate [mL/min]:							
ID = 1.0 mm	0.04	0.07	0.10	0.13	0.17	0.20	0.24
ID = 2.1 mm	0.15	0.3	0.45	0.6	0.75	0.9	1.05
ID = 4.6 mm	*0.7*	*1.4*	*2.1*	*2.8*	*3.5*	*4.2*	

Waters
THE SCIENCE OF WHAT'S POSSIBLE.

Overview

Characterisation by chemistry

Silica-based (oxides)

Organic polymer-based

others e.g. carbon

Characterisation by size

1960s	1970s	1980s	1990s	Now
>10 µm irregular	10 µm spherical	5 µm spherical	3–4 µm spherical	<3 µm spherical
Low pressure	1000 psi	3000 psi	6000 psi	9000–15,000 psi

Overview

Silica based stationary phases

porous **particles** non-porous

monoliths

Organic polymer based stationary phases

Silica Stationary Phases Changed in Surface Modification

First Ion Exchange Chromatography Papers: 1966

Horvath, C.G.; Lipsky, S. R. **Use of liquid ion exchange chromatography for the separation of organic compounds.** Nature (London, United Kingdom) (1966), 211(5050), 748-9. CODEN: NATUAS ISSN:0028-0836. CAN 65:75208 AN 1966:475208 CAPLUS

First RP HPLC Papers: 1976

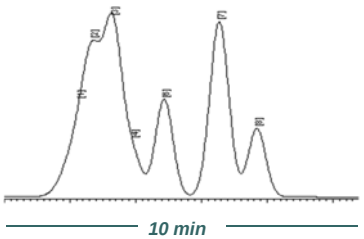
Chan, Henry W. S.; Levett, Gordon. **High performance liquid chromatography of oxygenated derivatives of unsaturated fatty acid esters.** Actes Congr. Mond. - Soc. Int. Etude Corps Gras, 13th (1976), Sect. D 73-9. CODEN: 36NUA6 CAN 87:163602 AN 1977:563602 CAPLUS

McCluer, Robert H.; Jungalwala, Firoze B. **High-performance liquid chromatographic analysis of glycosphingolipids and phospholipids.** Advances in Experimental Medicine and Biology (1976), 68(Curr. Trends Sphingolipidoses Allied Disord.), 533-54. CODEN: AEMBAP ISSN:0065-2598. CAN 86:135752 AN 1977:135752 CAPLUS

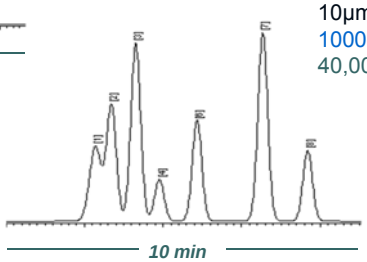
Koshy, K. T.; Vanderslik, A. L. **High-pressure liquid chromatographic method for the determination of 25-hydroxycholecalciferol in cow plasma.** Analytical Biochemistry (1976), 74(2), 282-91. CODEN: ANBCA2 ISSN:0003-2697. CAN 85:74432 AN 1976:474432 CAPLUS

Riedmann, M. **Elution strength and selectivity of the mobile phase in reverse phase high performance liquid chromatography (HPLC).** Fresenius' Zeitschrift fuer Analytische Chemie (1976), 279(2), 154-5. CODEN: ZACFAU ISSN:0016-1152. CAN 84:147086 AN 1976:147086 CAPLUS

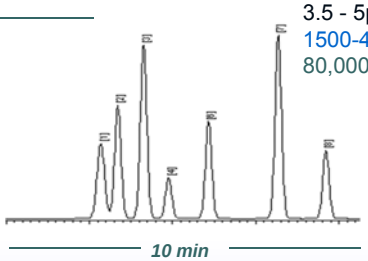
Silica Stationary Phases Changed in Shape



Late 1960's
40µm pellicular non-porous coated
100-500 psi (7-40 bar)
10,000 plates/meter



Early 1970's
10µm Irregular micro-porous
1000-2500 psi (70-180 bar)
40,000 plates/meter

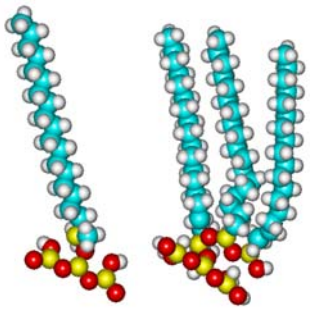


1980's to present day
3.5 - 5µm spherical micro-porous
1500-4000 psi (110-280 bar)
80,000 - 115,000 plates/meter

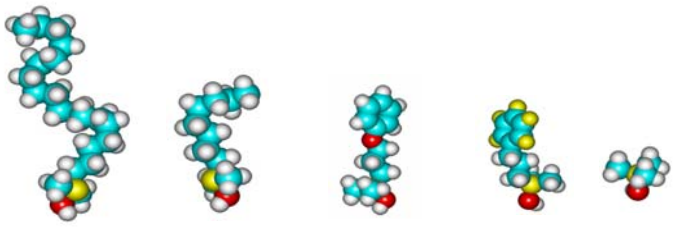
Surface Modified Silica

- Bonded phase shields polar silica surface, making it inaccessible for analyte molecules.
- Suppressing strong polar interactions with silica surface and substituting them with weak dispersive forces is a key factor of reversed-phase HPLC.
- Bonded phase types - C18, C8, C5, C1, Phenyl, CN, NH2, etc.

Monomeric and Polymeric Bonding C18-Silica

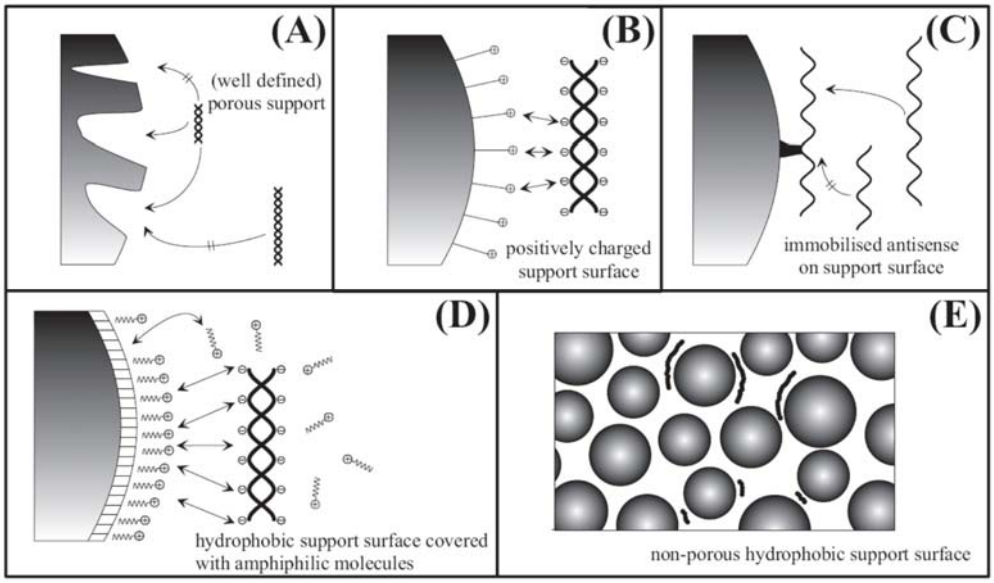


Selected Types of Bonded Ligands



C-18 C8 Oxy-Phenyl PFP C1

Other Separation Mechanisms



(A) Size Exclusion Chromatography (SEC), **(B)** Ion Exchange Chromatography (IEC), **(C)** Affinity Chromatography (AC), **(D)** Ion-Pair Reversed-Phase Chromatography (IP-RPC), **(E)** Slalom Chromatography (SC)

Silica vs Polymers

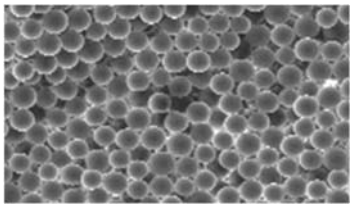
Silica

SEM photograph of NUCLEOSIL

+ high pressure stable
+ easy surface modification
+ high surface areas possible

- instable > pH 8.5
- tailing of basic analytes due to non functionalised silanolic groups possible

Polymers



SEM photograph of PS

+ high pH stability, both acidic and alkaline environment
+ easy surface modification
+ high surface areas possible
+ no free silanolic groups

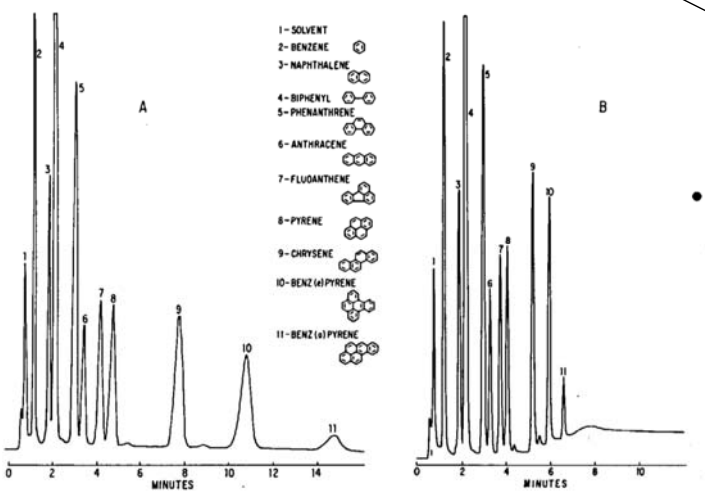
- lower pressure stability in comparison to silica

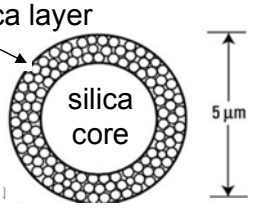
Porous Layer Beads

1955–1969 Synthesis of permanently bonded phases for gas chromatography (GC), preparative GC.

1965–1973 Synthesis of porous layer beads (Zipax), manufacture of microparticulate silica beads in collaboration with R.K. Iler **(Zorbax technology)** development of monolayer bonded phases based on Zorbax

1984–1992 Development of improved silica (Zorbax Rx, Type B silica) with improved chemical properties for chromatography of basic compounds). Development of steric-protected and bidentate silanes for improved bonded-phase stability at low and high pH (Collaboration with Joe Glajch).





- Reverse-phase separation of aromatic hydrocarbons. Column, 25 cm X 0.32 cm i.d., PSM-50/C_{18} (18 % C), 7 µm; temperature, 50 °C; pressure, 2000 psi; flowrate, 1.4 ml/min.; UV detector, 0.32 A UFS; samples, 10 µl of test mixture in methanol.
  A. Isochratic: mobile phase, 87.5 % methanol/water
  B. Gradient elution: mobile phase, linear gradient, 62.5 % methanol/water to 100 % methanol at 6 %/min.

J. J. Kirkland

J. J. Kirkland. Chromatographia, Vol. 8, No. 12, December 1975

Macro- and Meso-Porous Silica

Klaus Unger developed macroporous, controlled porosity, 10 µm silica particles

Unger K K.; Kern R; Ninou, M C.; Krebs K F. **High performance gel permeation chromatography with a new type of silica packing material.** Journal of Chromatography (1974), 99 435-43.

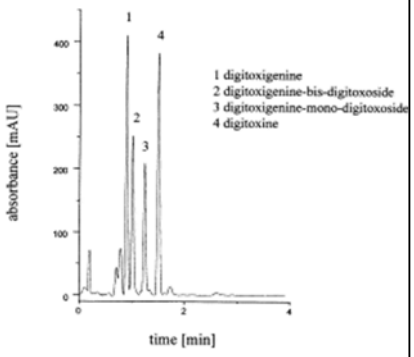
Synthesis of tailored porous oxides, in particular of silica, and alumosilicates with controlled pore structure and morphology

Unger, K.; Schick-Kalb, J.; Straube, B. **Porous silicon dioxide with variable and reproducible pore space structure from poly(ethoxysiloxane).** Colloid and Polymer Science (1975), 253(8), 658-64.

Synthesis of ordered mesostructured silicas as nanoparticles and agglomeration to hierarchically ordered beads of controlled size and pore structure

Lefevre, B.; Galarneau, A.; Iapichella, J.; Petitto, C.; Renzo, F. Di; Fajula, F.; Bayram-Hahn, Z.; Skudas, R.; Unger, K.. **Synthesis of Large-Pore Mesostructured Micelle-Templated Silicas as Discrete Spheres.** Chemistry of Materials (2005), 17(3), 601-607.

Klaus Unger

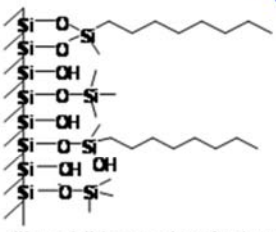


spherical porous silicas in the micron and submicron size range

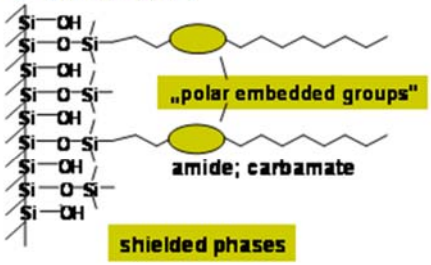
Chemically Modified Stationary Phases

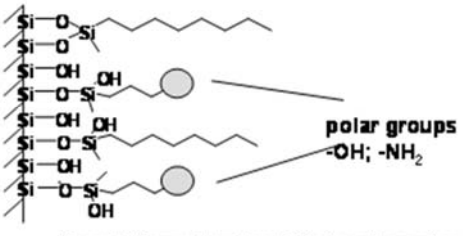
H. Engelhardt

Types of RP Columns

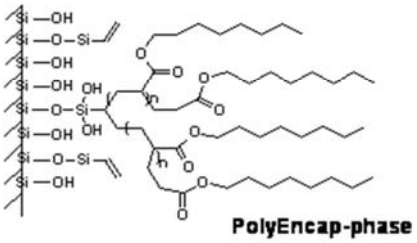


"brush" type; classical endcapping



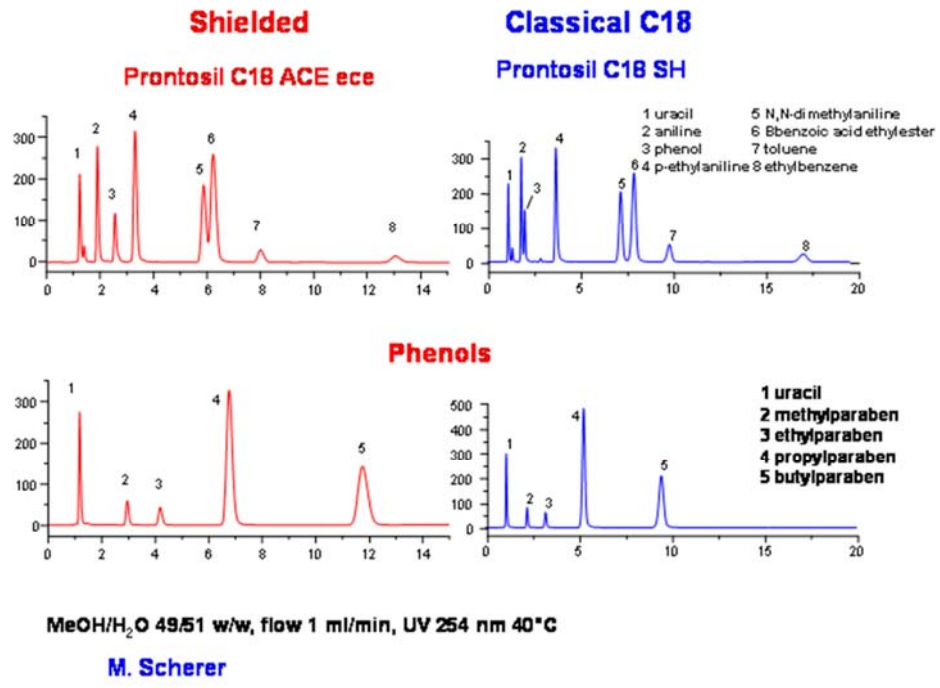


"brush"type; "hydrophilic" endcapping

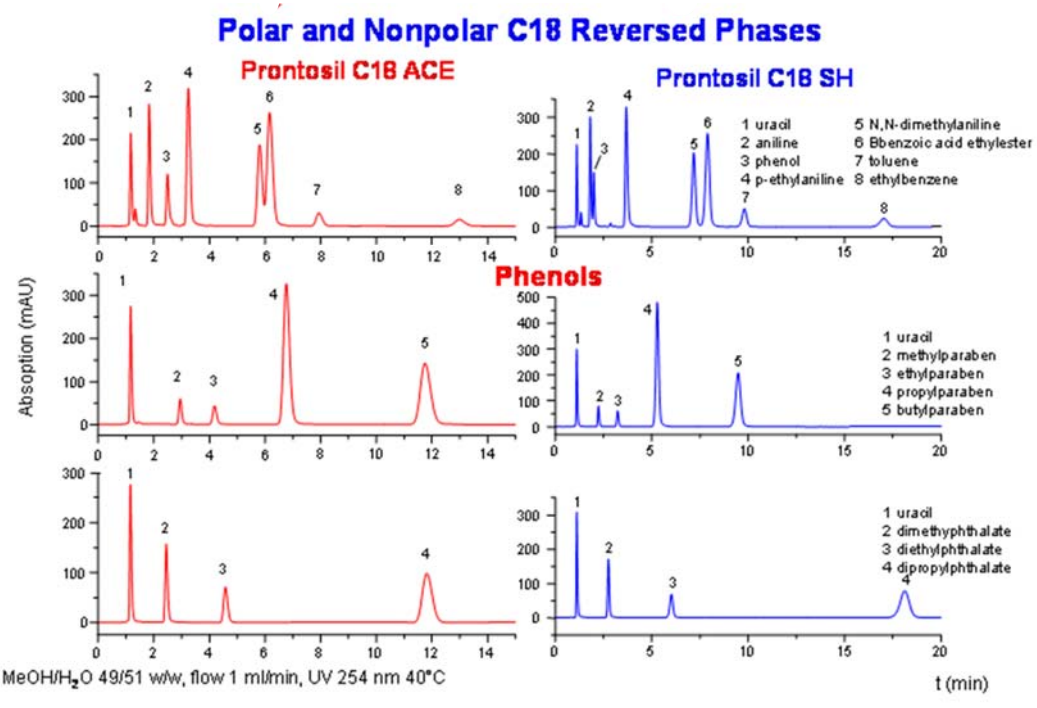


PolyEncap-phase

Chemically Modified Stationary Phases

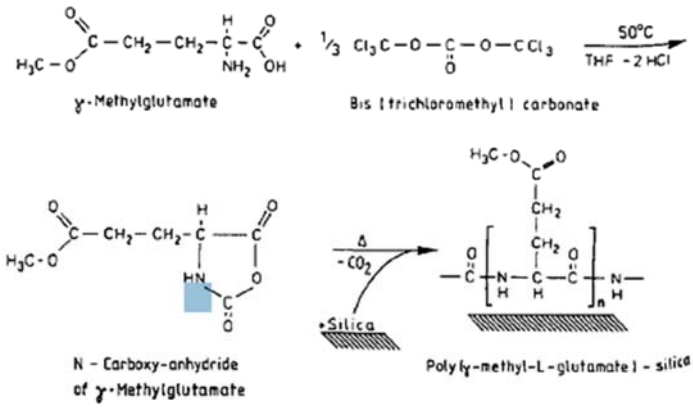


Chemically Modified Stationary Phases

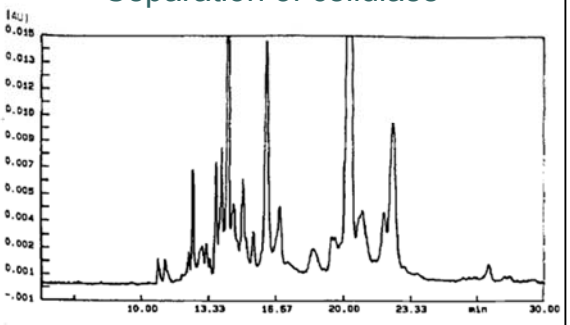


Surface Modification of Silica

Polymethylglutamate coated Silica

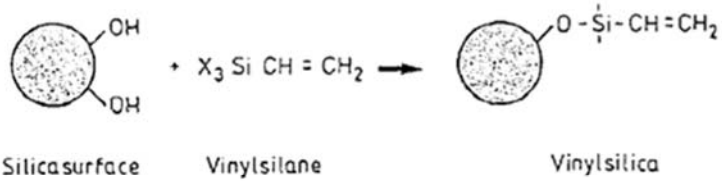


Separation of cellulase



Bentrop, D.; Kohr, J.; Engelhardt, H.Chromatographia (1991), 32(3-4), 171-8.

Polymer Encapsulated Stationary Phases



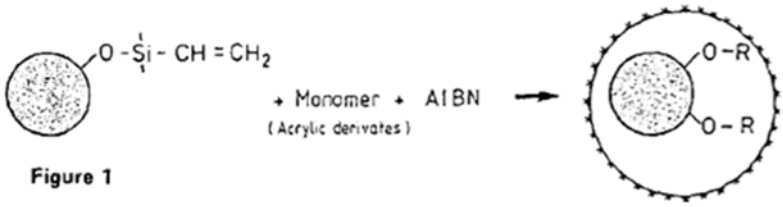
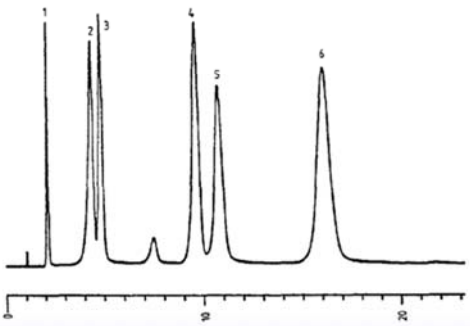


Figure 1

Separation of aromatic compounds



Engelhardt, H.; Löw, H.; Eberhardt, W.; Mauss, M. Chromatographia (1989), 27(11-12), 535-43.

Problems with TiO_2 and ZrO_2

Different optimization strategies required compared to silica based phases

Amphoteric surface properties

High packing densities but small surface area

Knowledge of classical RP chromatography not transferable

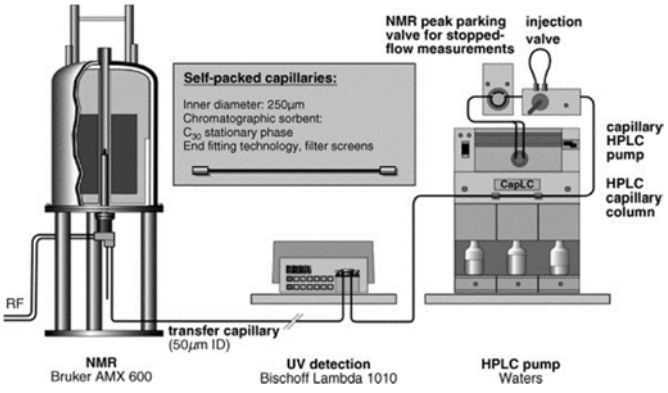
It's a different world

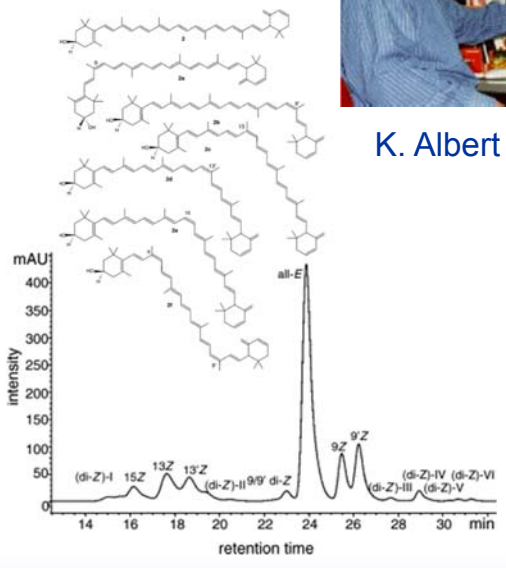
Silica Modification and Hyphenation Techniques

Development of new stationary phases for HPLC
with special focus on carotinoids e.g. C-30
Characterisation of stationary phases by Solid State NMR
Hyphenation of HPLC and GC to NMR

K. Albert

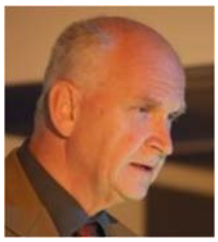
Set-up of the capHPLC–NMR (600 MHz) system





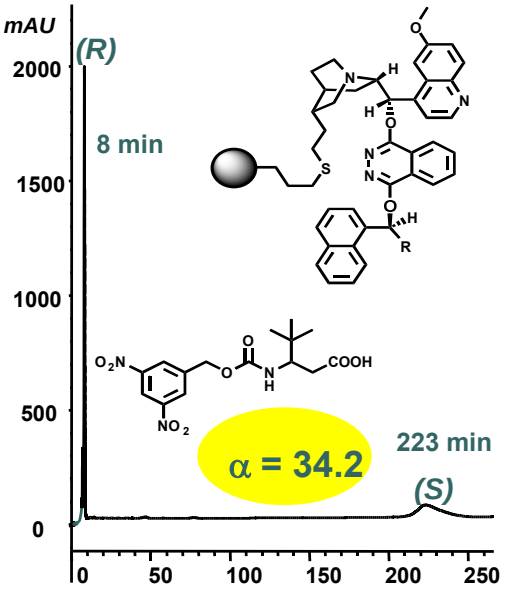
P. Hentschel et al.,Journal of Chromatography A, Vol. 1112, 1-2 (2006), 285-292

Enantionmers Separation



W Lindner

The development of novel synthetic selectors (receptors) useful for enantioselective separation techniques lies at the interface of organic, analytical and biological chemistry



Chromatographic conditions: Dim.: 100 x 4 mm ID
MeOH / H2O = 80 / 20 (v/v), 1 M NH4OAc total,
pHa = 6.0; 0.7 ml / min, 254 nm; 25°C.

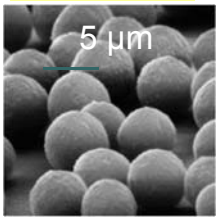
Materials for Highly Selective Molecular Recognition

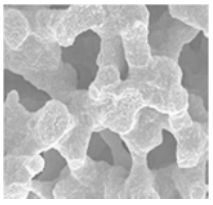
macroscopic → microscale → nanoscale → function

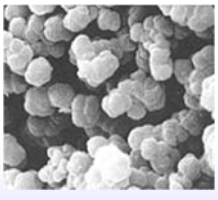
particles (powder)

monoliths (rods)

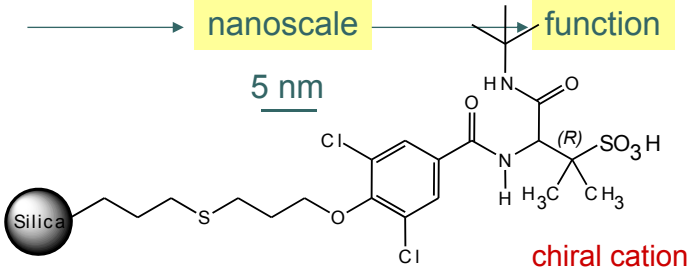
(disks)



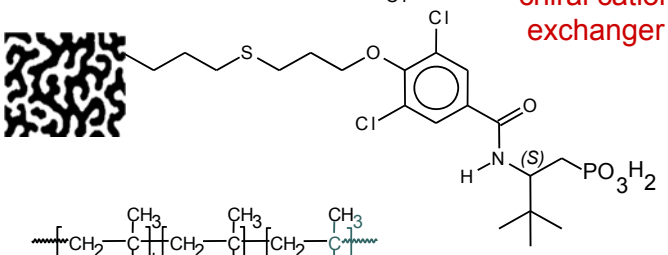


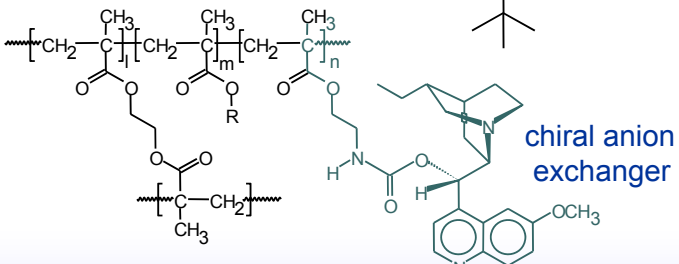


5 nm



chiral cation exchanger



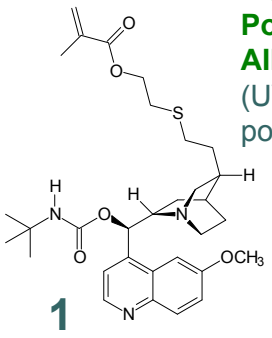


chiral anion exchanger

Capillary *Separation Technologies*

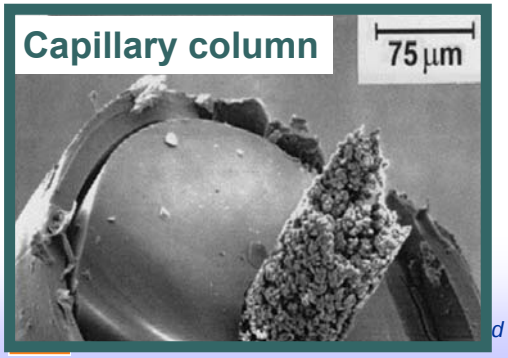
poly(**1**-co-HEMA-co-EDMA)

Monomers: 40 wt%
Porogens: 60 wt%
AIBN
(UV or thermal polymerization)

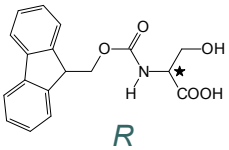


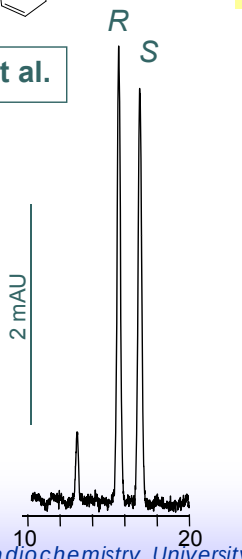
1

M. Lämmerhofer et al.



- nano-HPLC
- CEC
- µ-chip

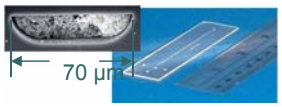




Trends in Anal. Chem.:

- **Miniaturization**
- **Nano-flow**
- **Minimal sample consumption**
- **MS compatibility**

- In-situ prepared organic polym.
- monolithic capillary columns
- single-step preparation
- macroporous, no frits
- flexible tailoring of
  - chemistry
  - morphology
  - porosity

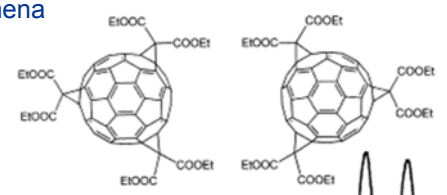


µ-Chip

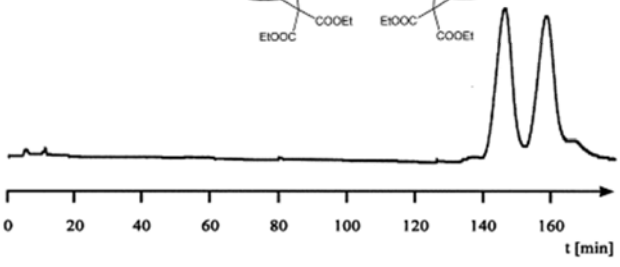
Chiral Separation

V. Schurig

- Gas-chromatographic Enantiomer Separation
- Analysis of Chiral Insect Pheromones and Flavours
- Chiral Electrochromatography
- Monoliths in Electrochromatography
- Unified Enantioselective Chromatography
- Enantiomerization
- Stopped Flow Gas Chromatography
- Enantioselective Coalescence Phenomena
- Chiral Environmental Organochlorines
- Enantioselective Antibodies
- Enantioselective Sensors
- Chiral Immunosensor
- Exochirality



B. Gross, J. Chromatogr. A 791 (1997) 65 –69, Enantiomer separation of [60]fullerene derivatives by micro-column high-performance liquid chromatography using (R)-(−)-2-(2,4,5,7-tetranitro-9-fluorenylideneaminooxy) propionic acid as chiral stationary phase.

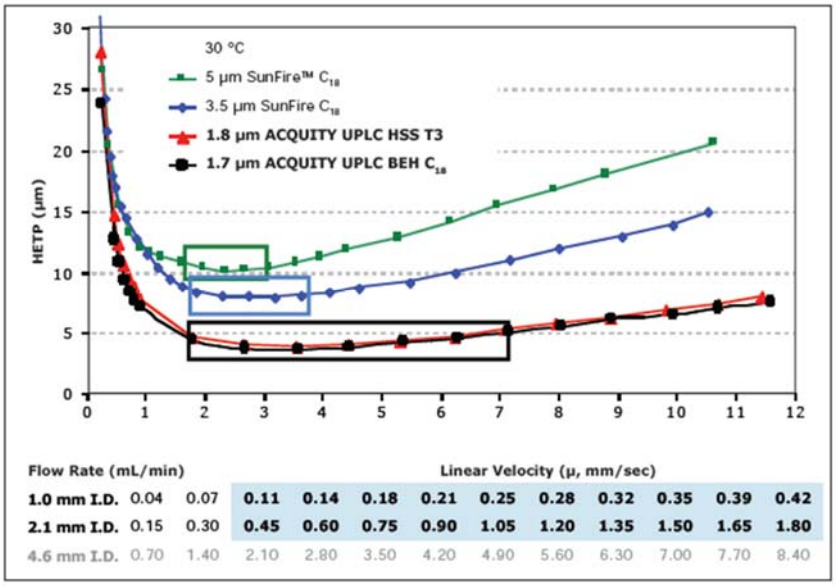


Enantiomer separation conditions: acetonitrile–water (75:25, v/v); flow-rate 0.4 µl/min.

UHPLC

Ultra High Performance Liquid Chromatography

Van Deemter Plott



Flow Rate (mL/min)			Linear Velocity (µ, mm/sec)									
1.0 mm I.D.	0.04	0.07	0.11	0.14	0.18	0.21	0.25	0.28	0.32	0.35	0.39	0.42
2.1 mm I.D.	0.15	0.30	0.45	0.60	0.75	0.90	1.05	1.20	1.35	1.50	1.65	1.80
4.6 mm I.D.	0.70	1.40	2.10	2.80	3.50	4.20	4.90	5.60	6.30	7.00	7.70	8.40

Waters
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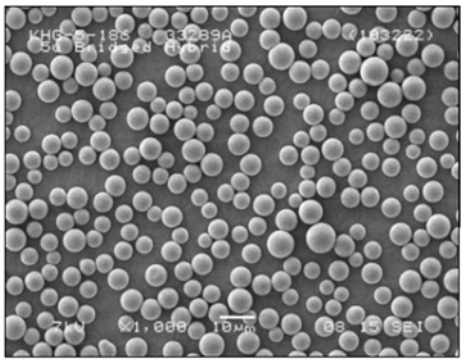
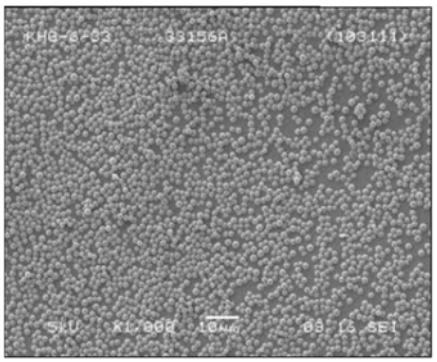
Particle Sizes



Conventional HPLC material, 5 µm

Silica particles



UHPLC material, 1.7 µm

Bridged Ethyl Hybrid Particles (BEH)

Why Ultra High Performance Liquid Chromatography (UHPLC)?

Plate count N is inverse proportional to particle size

$$N \propto 1/d_p$$

d_p = particle size

↓

Resolution Rs is proportional to square root of N

$$Rs \propto \sqrt{N}$$

↓

Resolution Rs is inverse proportional to square root dp

$$Rs \propto \sqrt{1/d_p}$$

Backpressure increases when particle size decreases

$$p_{back} \propto \frac{1}{d_p}$$

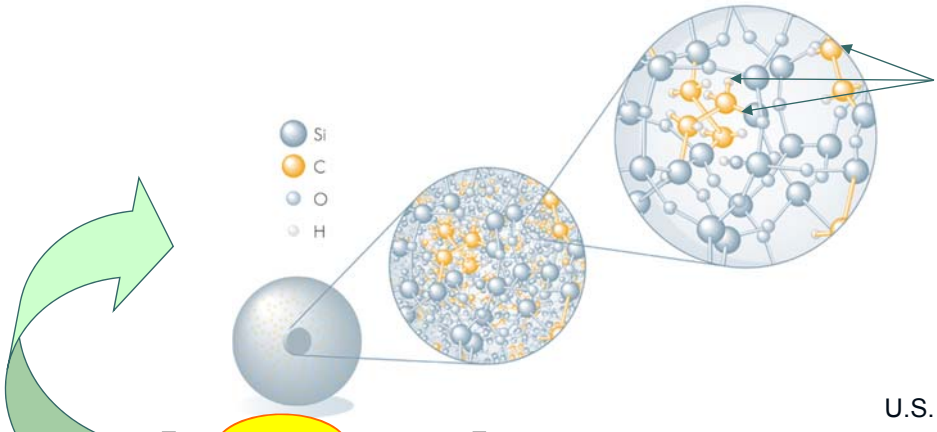
→ **Small particles have higher backpressure**

UHPLC Systems and columns have to be robust to withstand pressure between 800 and 1200 bar

Acquity UPLC (Waters)®

Agilent® 1290

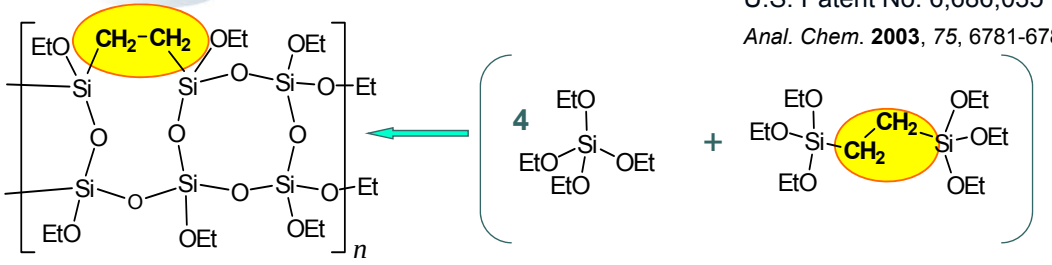
Bridged Ethyl Hybrid Particles



Bridged Ethyl
In Silica Matrix

U.S. Patent No. 6,686,035 B2

Anal. Chem. **2003**, *75*, 6781-6788



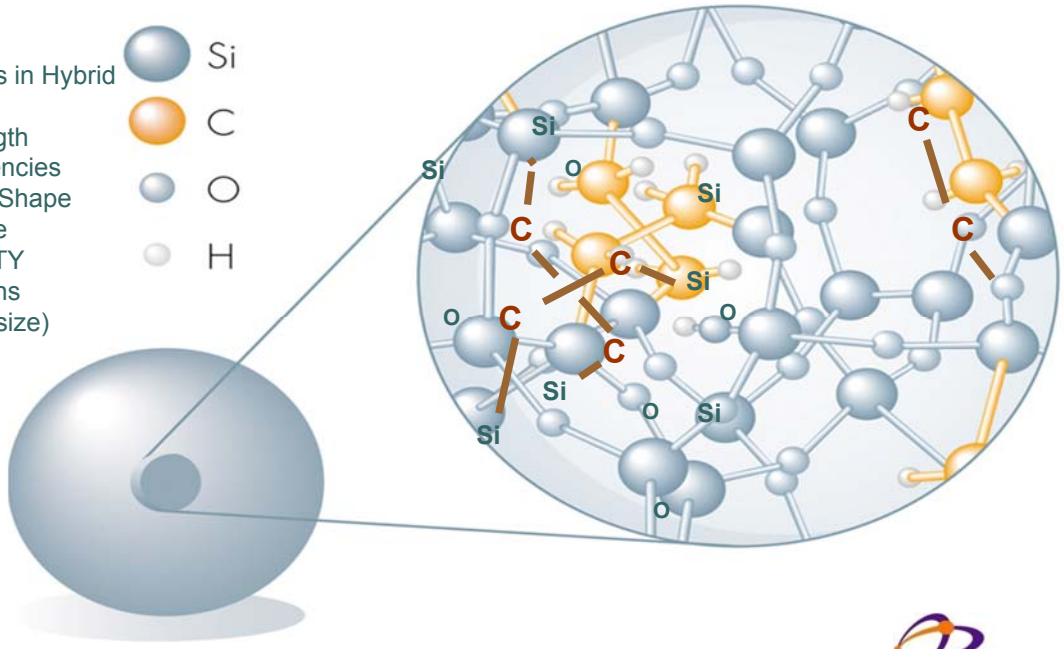
Polyethoxysilane (BPEOS)

Tetraethoxysilane (TEOS)

Bis(triethoxysilyl)ethane (BTEE)

Bridged Ethyl Hybrid Particles (BEH)

Bridged Ethanes in Hybrid Matrix
Improved Strength
Improved Efficiencies
Improved Peak Shape
Wider pH Range
Used in ACQUITY UPLC™ Columns
(1.7µm particle size)



BEH Technology™
Waters
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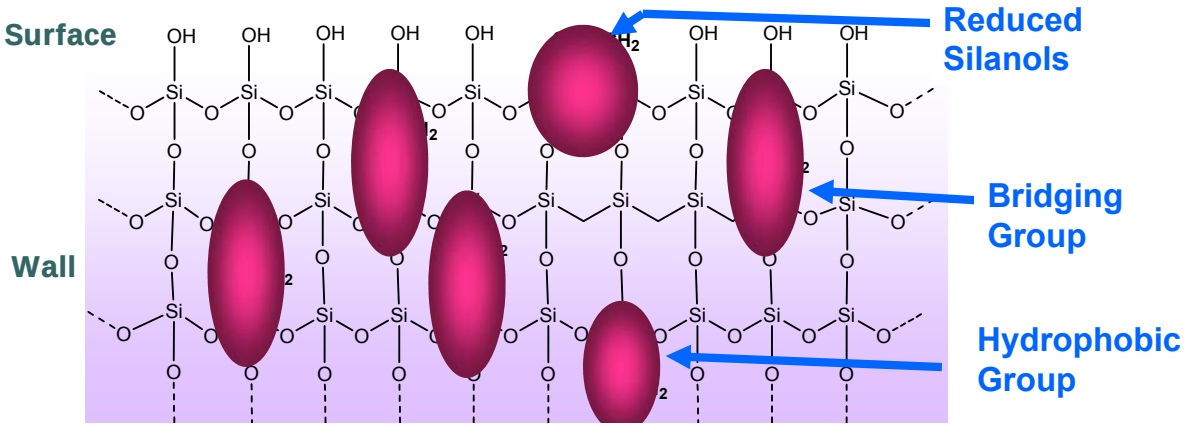
BEH Technology™ Particle Attributes



Hybrid Particle Attribute	RP-HPLC Consequence
Surface hybrid groups reduce surface silanol concentration	Reduced USP peak tailing factors for bases
Internal bridging groups provide high interconnectivity	Increased chemical & mechanical stability
Internal hybrid groups provide hydrophobicity	Increased high pH stability of column

Waters
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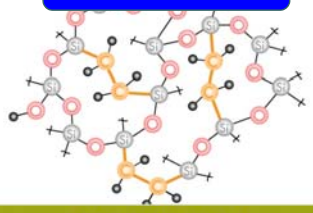
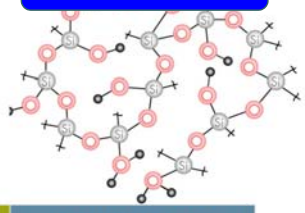
Silica and Hybrid Materials

Hybrid Material

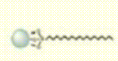
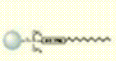
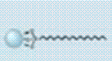


Silica Material



	ACQUITY UPLC BEH Columns		ACQUITY UPLC HSS Columns
Particle Type	Ethylene Bridged Hybrid (BEH)		High Strength Silica (HSS)
Particle Size	1.7 µm		1.8 µm
Maximum Rated Pressure	15000 psi (~1000 bar)		15000 psi (~1000 bar)
Pore Diameter/Volume	130Å / 0.7 mL/g	300Å / 0.7 mL/g	100Å / 0.7 mL/g
Surface Area	185 m^2/g	90 m^2/g	230 m^2/g
Available Chemistries	C_{18}, C_8, Shield RP18, Phenyl, HILIC	C_{18}, C_4	C_{18}, C_{18} SB, T3
pH Range	1-12; (RP18: 2-11); (HILIC: 1-8)	C_{18}: 1-12; C_4: 1-10	C_{18}: 1-8; C_{18} SB, T3: 2-8

Available Column materials

	BEH Particle					HSS Particle		
Chemistry	C_{18}	C_8	Shield RP18	Phenyl	HILIC	C_{18}	C_{18} SB	T3
								
Ligand Type	Trifunctional C_{18}	Trifunctional C_8	Monofunctional Embedded Polar Group	Trifunctional C_6 Phenyl	–	Trifunctional C_{18}	Trifunctional C_{18}	Trifunctional C_{18}
Ligand Density*	3.1 µmol/m^2	3.2 µmol/m^2	3.3 µmol/m^2	3.0 µmol/m^2	–	3.2 µmol/m^2	1.6 µmol/m^2	1.6 µmol/m^2
Carbon Load*	18%	13%	17%	15%	–	15%	8%	11%
Endcap Style	Proprietary	Proprietary	TMS	Proprietary	–	Proprietary	None	Proprietary
pH Range	1-12	1-12	2-11	1-12	1-8	1-8	2-8	2-8

Hybrid Material

Silica Material

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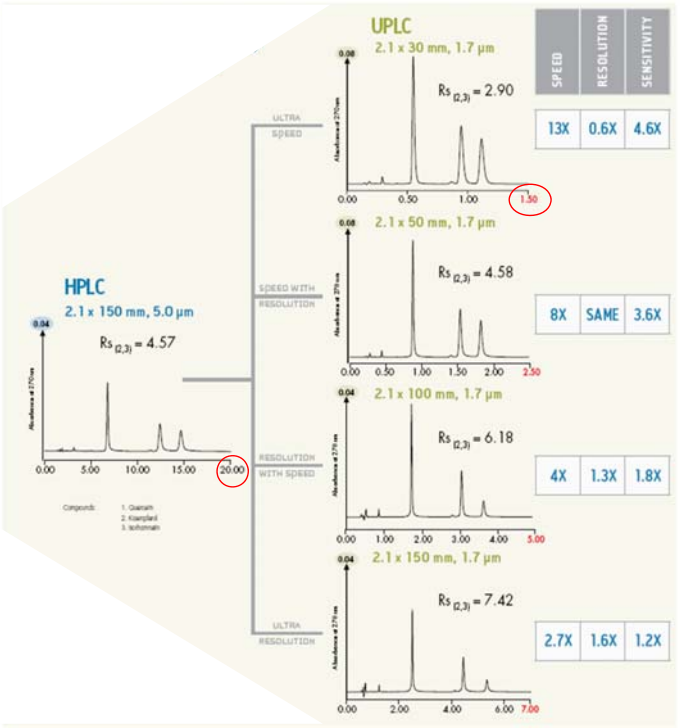
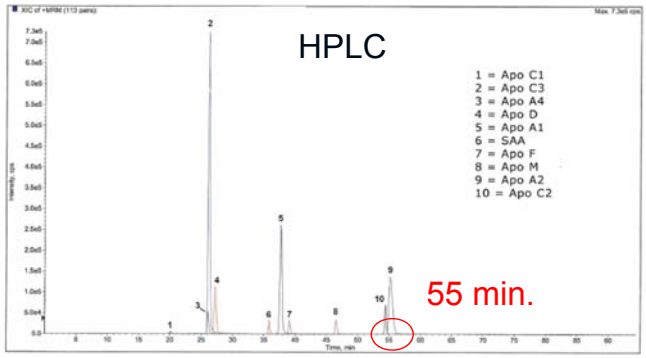
Seperations of Small Molecules

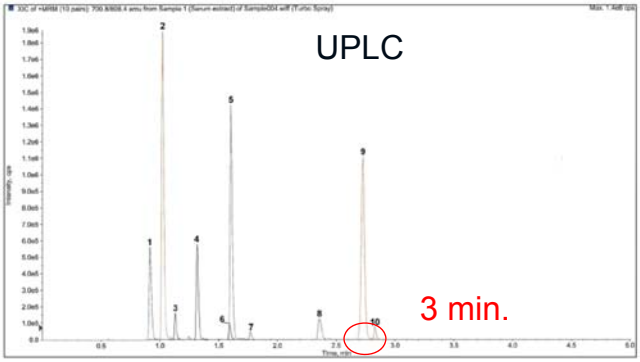
1. Quercetin
2. Kaempferol
3. Isorhamnetin



Application in Proteomics - Apolipoproteins



Time (min)	%A
0	100
3	100
5	95
60	40
70	10
82.5	10
82.6	100
95	100



Time (min)	%A
0	10
3	45
3.1	90
4	90
4.1	10
5	10

Overview

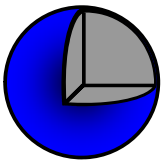
Silica based stationary phases

porous **particles** non-porous

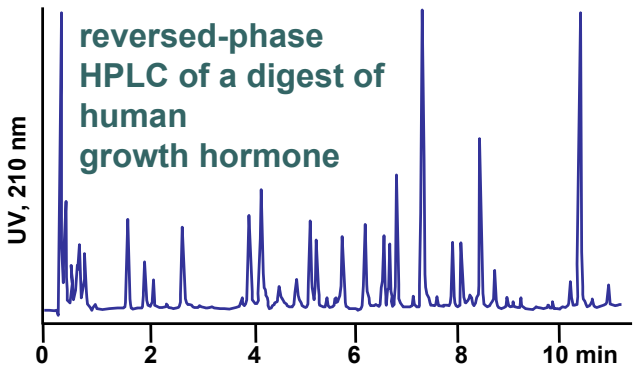
Micropellicular Stationary Phases

Cs. Horváth
1930-2004

"The significance of rapid HPLC is expected to have great potential in obtaining quick analytical information"
Cs. Horváth, 1988

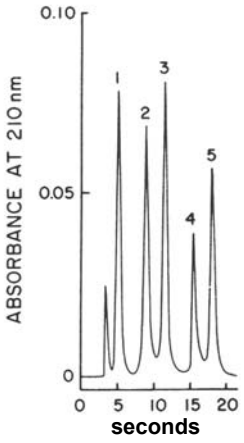


Micropellicular particle

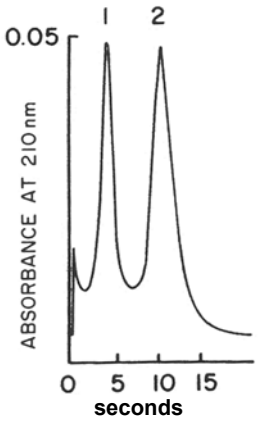


Unger, Jilge, Kinkel, Hearn, J. Chromatogr. 359 (1986) 61
Kalghatgi and Horváth, J. Chromatogr. 443 (1988) 343)

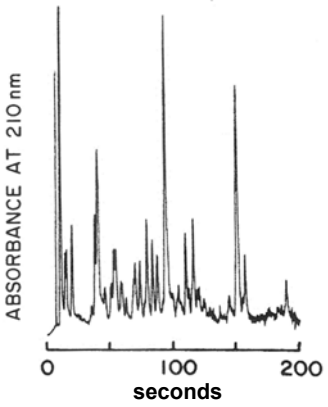
Micropellicular Sorbents for Rapid Reversed-Phase Chromatography of Proteins and Peptides.



Separation of protein mixture



Separation of β-Lactoglobulin B and A



Separation of tryptic digest of methionyl human growth hormone

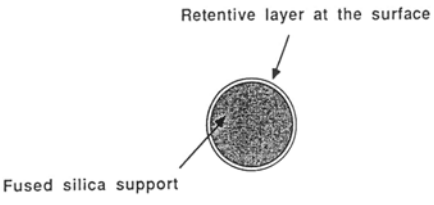
Kalghatgi, Krishna; Horvath, Csaba. **Rapid peptide mapping by high-performance liquid chromatography.** Journal of Chromatography (1988), 443 343-54.

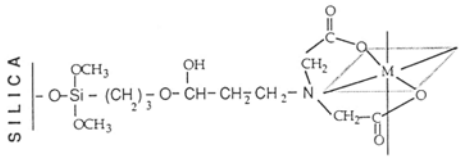
Molnar I; Horvath C, **Separation of amino acids and peptides on non-polar stationary phases by high-performance liquid chromatography**. Journal of chromatography (1977), 142 623-40.

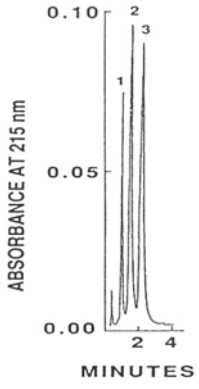
El Rassi, Z; Horvath, C, **Hydrophobic interaction chromatography of t-RNA's and proteins.** Journal of Liquid Chromatography (1986), 9(15), 3245-68.

Affinity Chromatography Based on Micropellicular Silica

Rapid metal interaction chromatography of proteins on Co^{+2}/IDA columns







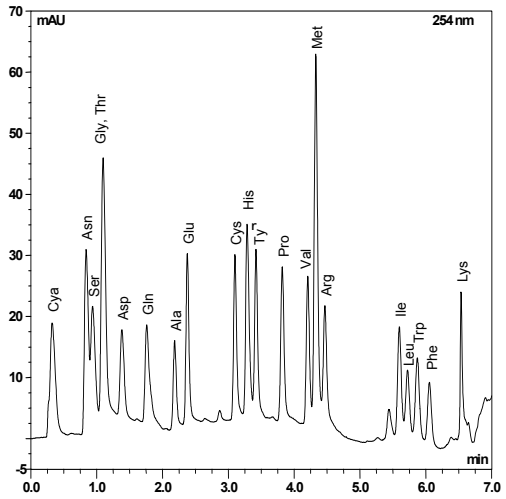
Bonn, G. K.; Kalghatgi, K.; Horne, W. C.; Horvath, C.. **Rapid metal-interaction chromatography of proteins and peptides on micropellicular sorbents.** Chromatographia (1990), 30(9-10), 484-8.

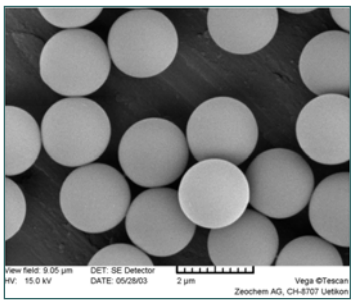
El Rassi, Ziad; Truei, Yunghuoy; Maa, Yih Fen; Horvath, Csaba. **High-performance liquid chromatography with concanavalin A immobilized by metal interactions on the stationary phase.** Analytical Biochemistry (1988), 169(1), 172-80.

Corradini, D; El Rassi, Z; Horvath, C; Guerra, G; Horne, W, **Combined lectin- affinity and metal-interaction chromatography for the separation of glycophorins by high-performance liquid chromatography.** J. Chromatography (1988), 458 1-11.

Non-Porous Silica

separation of amino acids





KOVASIL MS 1.5 µm Transmission Electron Microscopy

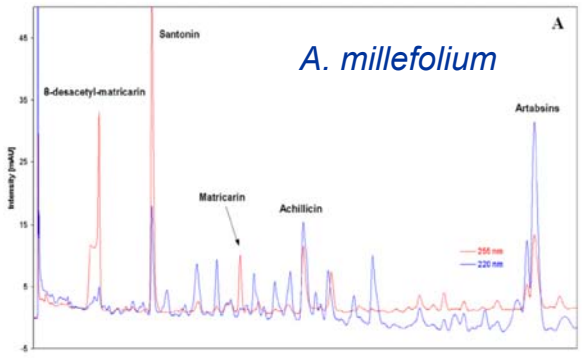
Separation of PTH-amino acids

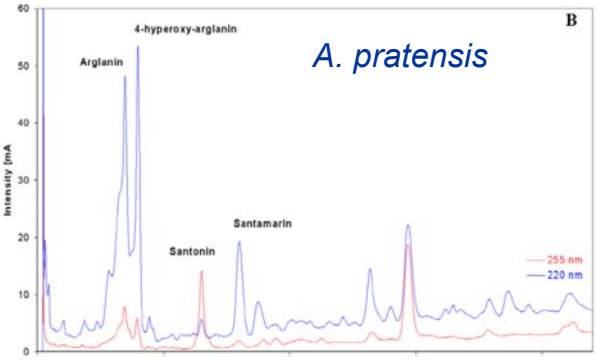
Column: 33 x 4.6 mm; **Kovasil MS-C14**
Eluents: A: Water + 0.1% HFBA
B: 90% acetonitrile-10% water + 0.8% HFBA
Gradient: 0-3.55 min to 12% B; 3.55-6.55 min to 20% B; 6.55-6.65 min to 100% B; 6.65-7.00 min 100% B.
Flow rate: 1.0 ml/min
Detection: 254 nm
Temperature:25°C

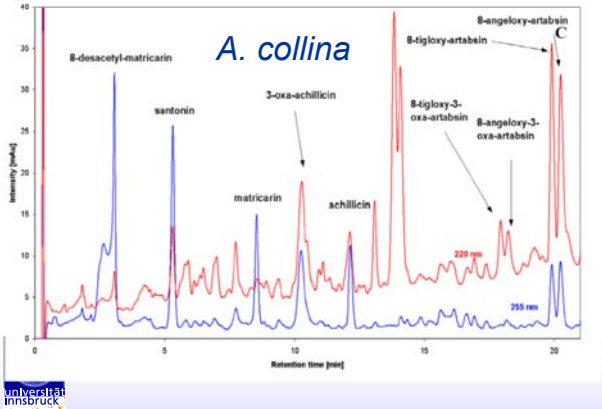
B. Boros, K. Kovasc and R. Ohmacht. ***Fast separation of amino acid phenylthiohydantion derivatives by HPLC on a non-porous stationary phase****. 51, 2000, S202-204.*

Institute of Analytical Chemistry and Radiochemistry, University of Innsbruck

Non-porous Silica







Column:
Kovasil MS C18, 33x4.6 mm I.D.
Eluent:
A: 2% CH3CN + 98% H2O
B: 95% CH3CN + 5% H2O
*Flow rate:*1.0 ml/min
Temperature: ambient

ESI-MS
Probe temp: 250ºC, *Probe:* 3.5kV
AQA max: 100V, +ve 10V, +ve 70V, +ve 100V

Robert Ohmacht, Pecs University

Institute of Analytical Chemistry and Radiochemistry, University of Innsbruck

Overview

Silica based stationary phases

porous **particles** non-porous

monoliths

Monolithic Silica Columns

Available online at www.sciencedirect.com

ScienceDirect

Journal of Chromatography A, 1191 (2008) 231–252

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Review

Preparation of monolithic silica columns for high-performance liquid chromatography

Oscar Núñez [a,b,*], Kazuki Nakanishi [c], Nobuo Tanaka [b]

[a] *Department of Analytical Chemistry, Faculty of Chemistry, University of Barcelona, Martí i Franquès 1-11, 08028 Barcelona, Spain*
[b] *Department of Biomolecular Engineering, Graduate School of Science and Technology, Kyoto Institute of Technology, Goisho-Kaido-cho, Matsugasaki, Sakyo-ku, Kyoto 606-8585, Japan*
[c] *Department of Chemistry, Graduate School of Science, Kyoto University, Kitashirakawa, Sakyo-ku, Kyoto 606-8502, Japan*

Available online 13 February 2008

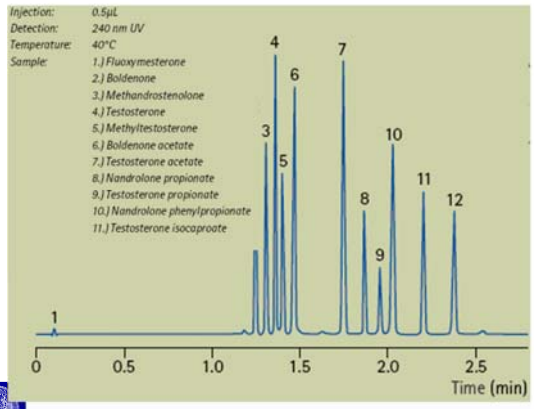
Development of monolithic silica columns consisting of continuous silica skeletons and throughpores of μm size.

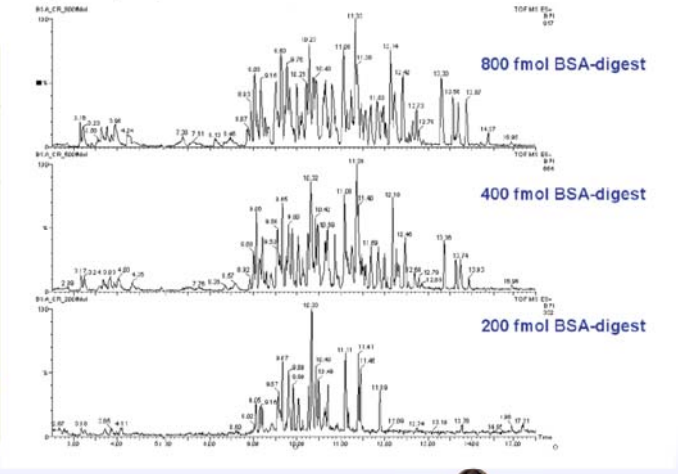
Particle packed columns and monolithic columns in high performance liquid chromatography-comparison and critical appraisal, K. K. Unger et al., J. Chromatgr. A. 1184 (2008), 393-415

Monolithic Silica Columns

Merck Chromolith HPLC Columns, Merck KGaA (Darmstadt, Germany)

SILICA MONOLITHS
Different chemistries (C-18, C-8, NP,…)
Different dimensions (0.05, 0.1, 0.2, 2, 3, 4.6 mm)
Different applications (bioanalytics, pharma-, food-environmental analytics,…)





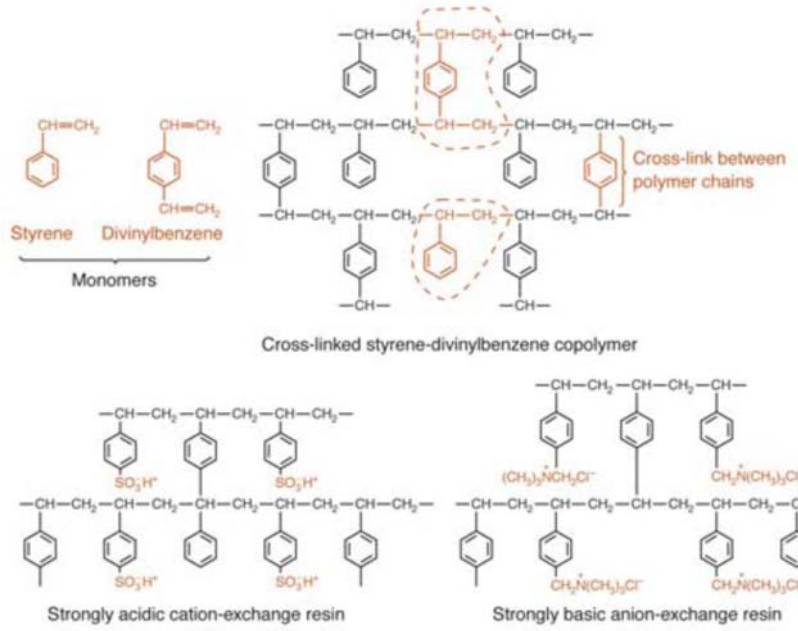
Overview

porous **particles** non-porous

monoliths

Organic polymer based stationary phases

PS/DVB Particles

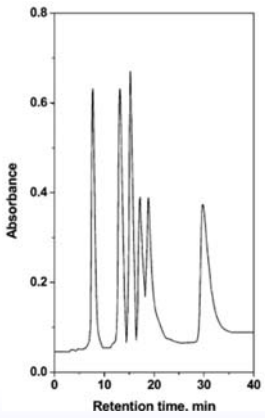


Porous polymer

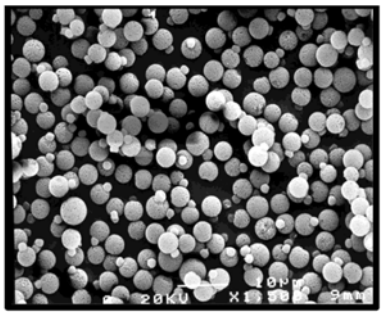
C. M. Cheng, F. J. Micale, J. W. Vanderhoff, M. S. El-Aasser, Synthesis and characterization of monodisperse porous polymer particles. Journal of polymer science Part A 30, 235-244, 1992.

Marina Galia, Frantisek Svec, Jean M. J. Frechet , Monodisperse polymer beads as packing material for high-performance liquid chromatography: effect of divinylbenzene content on the porous and chromatographic properties of poly(styrene-co-divinylbenzene) beads prepared in presence of linear polystyrene as a porogen.
Journal of Polymer Science, Part A: Polymer Chemistry 32, (11), 2169-75 1994

Xu, Mingcheng; Peterson, Dominic S.; Rohr, Thomas; Svec, Frantisek; Frechet, Jean M. J. Polar polymeric stationary phases for normal- phase HPLC based on monodisperse macroporous poly(2,3-dihydroxypropyl methacrylate-co-ethylene dimethacrylate) beads. Analytical Chemistry (2003), 75(4), 1011-1021.



Separation of aniline derivatives using column packed with 3-μm 70% diol stationary phase obtained from poly(glycidylmethacrylate-co-ethylene dimethacrylate) beads. Chromatographic conditions: column 150 4.6 mm; mobile phase, linear gradient of 15-40% THF in hexane in 40 min; flowrate 1 mL/min; UV detection at 254 nm.



Perfusion Chromatography

F. Regnier

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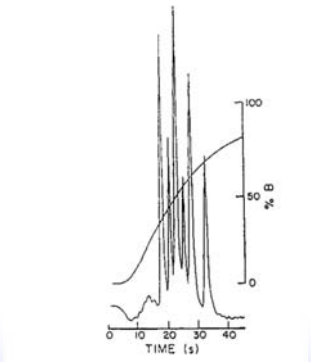
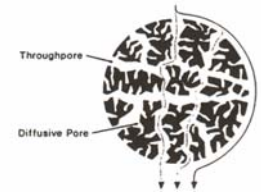
PERFUSION CHROMATOGRAPHY
AN APPROACH TO PURIFYING BIOMOLECULES

Liquid chromatography (LC) remains the preeminent technique for isolating and recovering proteins and peptides. Highly selective, extremely flexible, and very gentle, it provides

Perfusion chromatography is a technique based on fluid dynamics for reducing stagnant mobile phase mass transfer in liquid chromatography. This is achieved by using supports with large pores that allow mobile phase to flow through particles.





A rapid separation of standard proteins on Poros Packing material

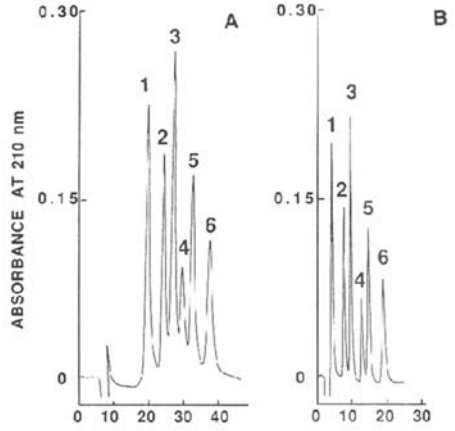
Overview

porous **particles** non-porous

monoliths

Organic polymer based stationary phases

PS/DVB Particles



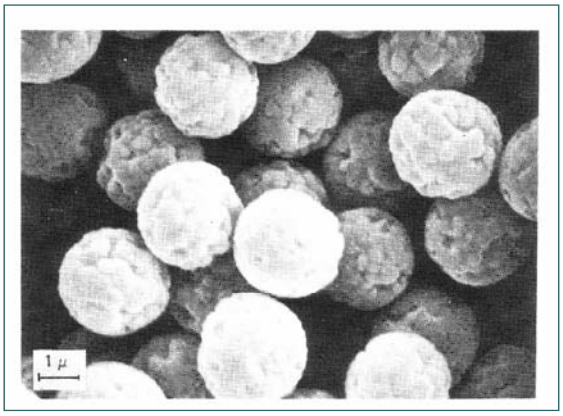
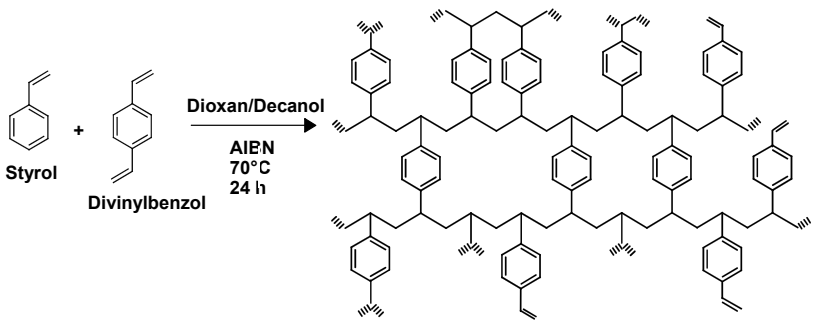


Fig. 4. Chromatograms of a protein mixture obtained on a 30 × 4.6 mm I.D. column under two different conditions. Flow-rate and temperature: (A) 3 ml/min, 25°C; (B) 7 ml/min, 80°C. Linear gradient in 1 min from 10 to 100% acetonitrile in water containing 0.2% TFA. Samples: 1, 100 ng of ribonuclease A; 2, 50 ng of cytochrome *c*; 3, 10 ng of lysozyme; 4, 100 ng of L-asparaginase; 5, 100 ng of *β*-lactoglobulin A; 6, 100 ng of ovalbumin.

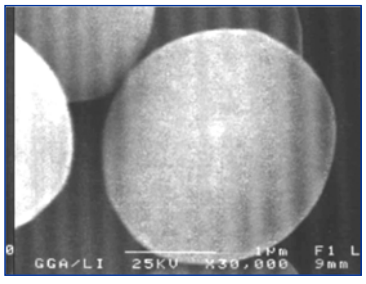
Maa, Yih Fen; Horvath, Csaba. **Rapid analysis of proteins and peptides by reversed-phase chromatography and polymeric micropellicular sorbents.** Journal of Chromatography (1988), 445(1), 71-86.

Synthesis of Polystyrol-Divinylbenzol Particles



Polystyrol-Divinylbenzol

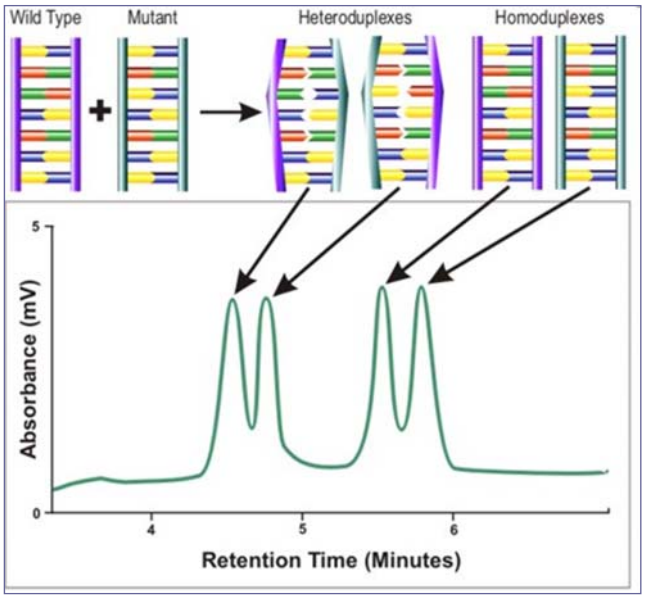
In situ polymerization of PS/DVB

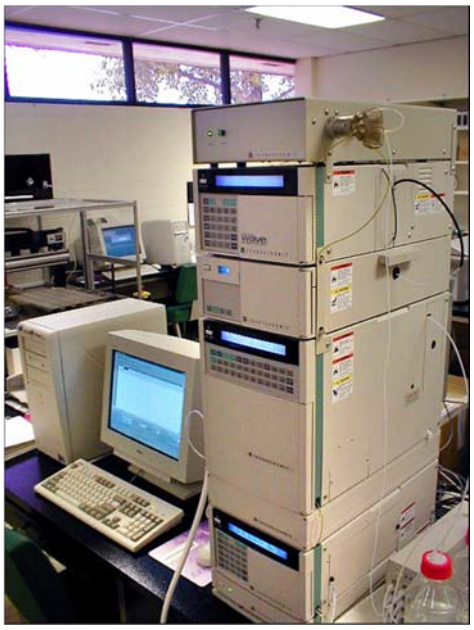


Non porous PS/DVB

G. Bonn, C. Huber. P. Oefner, Nucleic Acid Separation on alkylated non porous polymer beads, U.S. Patent No. 5,585,236

Transgenomic Wave-System - Mutation Analysis





Transgenomic Wave HPLC-System

Bonn, Gunther; Huber, Christian; Oefner, Peter. ***Separation of nucleic acid fragments with alkylated nonporous polymer beads.*** *PCT Int. Appl. (1994), 30 pp.*

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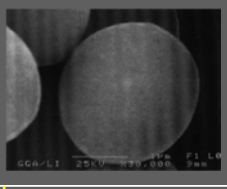
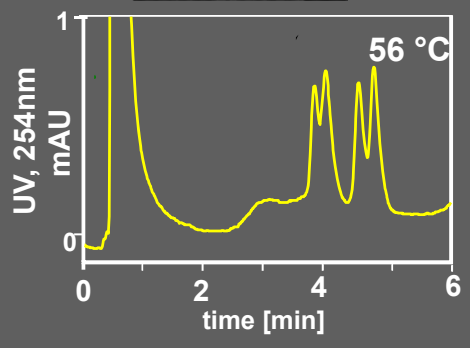
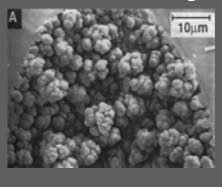
Mutation Detection by Denaturing HPLC using Packed Columns and Monolithic Capillaries

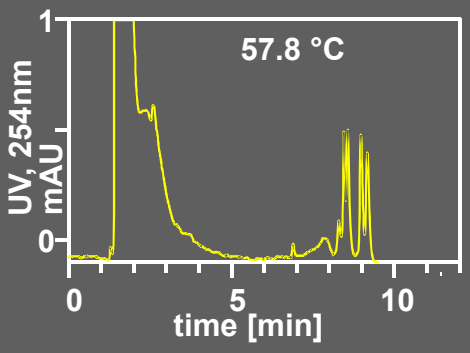
Packed Column





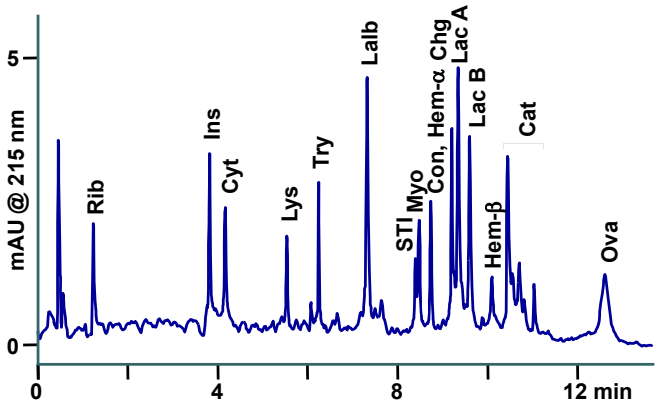
Monolithic Capillary

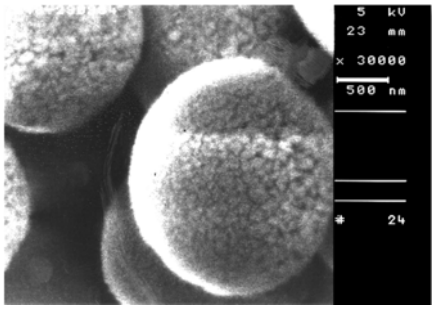




column:	50 x 4.6 mm I.D.	→	50 x 0.2 mm I.D.
flow rate:	1 ml/min	→	3.0 µl/min
injection vol.:	5 µl	→	500 nl

High-Efficiency Intact Protein Separation with C18-PS/DVB Particles

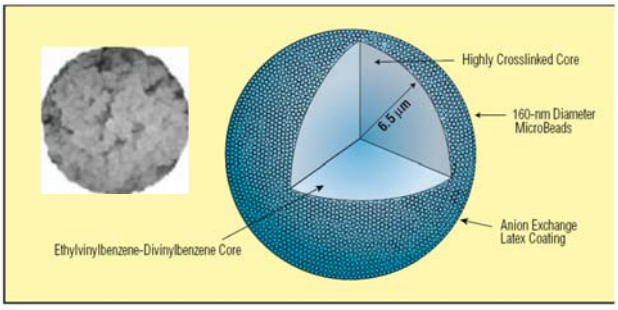




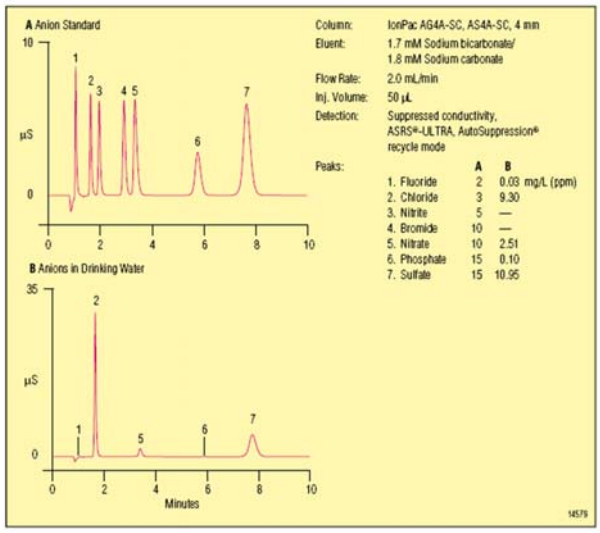
53 x 4 mm I.D., 2.3 µm PS-DVB-C18 particles, 38-54% acetonitrile in 0.2 % trifluoroacetic acid in 15 min, 560 µl/min, 80 °C.

Huber, Kleindienst, Bonn, Chromatographia 44 (1997) 438

Anion-Exchange Polymer

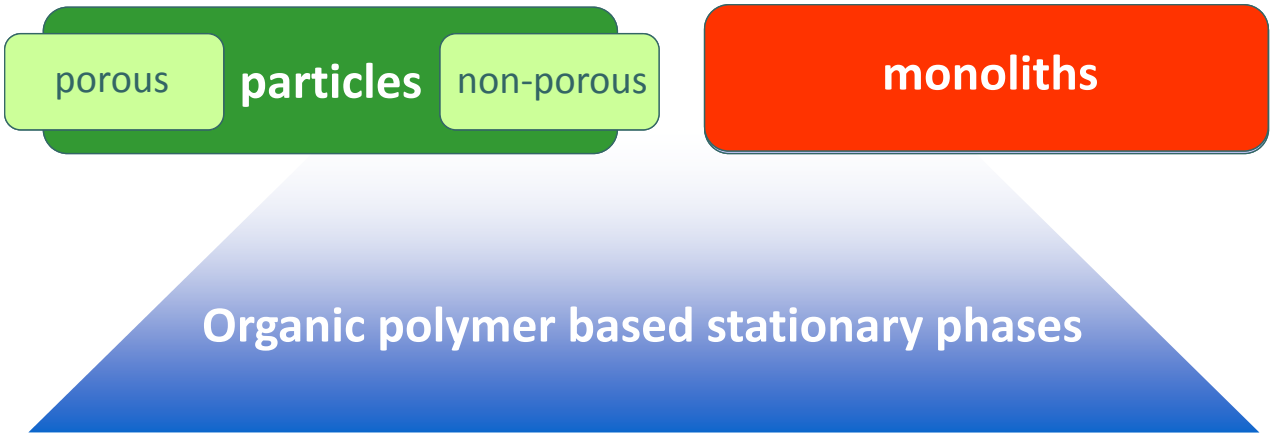


The IonPac AS4A-SC® packing is a structure composed of a highly crosslinked core and a MicroBead™ anion-exchange layer attached to the surface. The substrate for the IonPac AS4A-SC column is a 13-µm diameter microporous resin bead, consisting of ethylvinylbenzene crosslinked with 55% divinylbenzene.The anion exchange layer is functionalized with very hydrophilic quaternary ammonium groups.



Determination of inorganic anions in drinking water sample on an IonPac AS4A-SC column

Overview



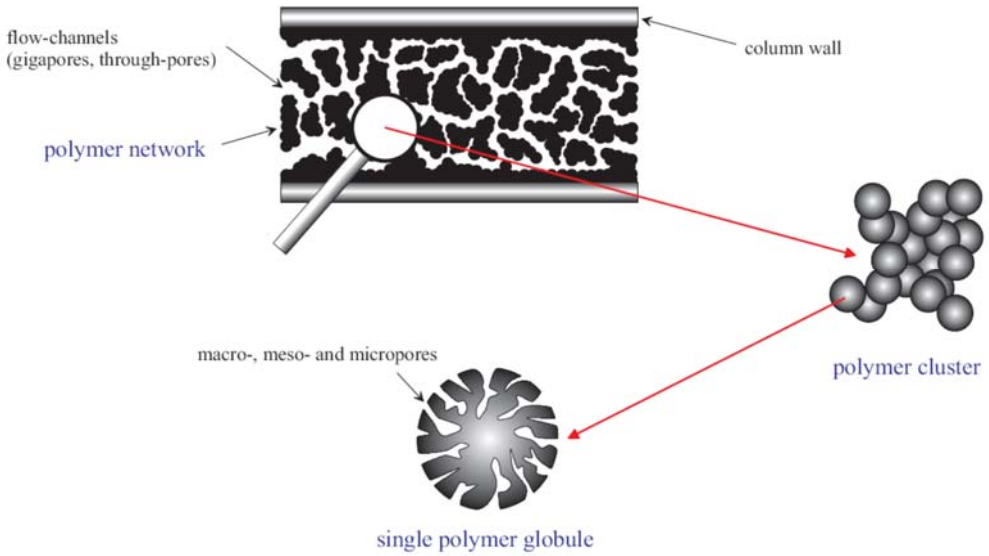
Historical Abstract

- **1950s, Robert Synge** was the first to postulate polymer structures, which were similar to what is defined as monolith today
- **1967, Kubin et al.,** polymerization in a glas column, poly(2-hydroxy-ethyl methacrylate-co-ethylene dimethacrylate)
  → low permeability and poor efficiency
- **1970,** foam filled columns for GC and HPLC (open pore polyurethane)
  → insufficient mechanical stability, strong swelling
- **1980, Hjerten et al.,** introduced the concept of compressed gels (***continuous beds***) - copolymerisation of acrylic acid and N,N'-methylene bisacrylamide (efficient separation of proteins) ***"stationary phases with reduced discontinuity"*** (for single piece polymer)
- **mid-1980, Tennikova et al.,** short monolithic separation beds (copolymerisation of glycidyl methacrylate as monomer and high amounts of ethylene dimethacrylate as crosslinker) - arranged in a pile or sliced into disks

Historical Abstract

- **Svec et al.,** extended the process to conventional HPLC column housings (8 mm I.D.), rigid macroporous polymers with cylindrical shape ***„continuous polymer rods“***
- Since **1992**, a vast variety of rigid organic monolithic stationary phases with different chemistry, functionality and column geometry has been reported for HPLC
- **1993**, Kennedy et al. **The term monolith**, which probably is the most common expression for the new class of macro-porous polymers, **was first introduced** to describe a single piece of derivatised cellulose sponge for the fractionation of proteins, but rapidly found general acceptance.

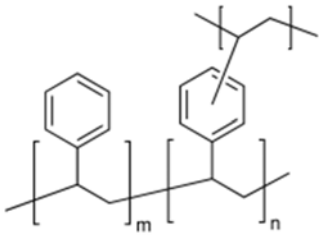
Monolithic Structure



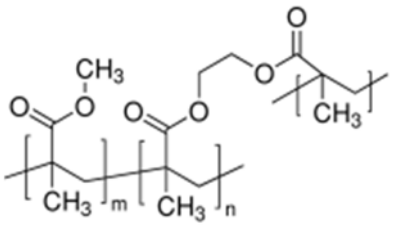
Schematic representation of the structure and the morphology of a typical monolithic polymer, prepared in a column housing as an unstirred mold.

Organic Monoliths

Styrene monoliths have been prepared by thermally (AIBN or benzoyl peroxide) initiated copolymerisation of styrene and divinylbenzene to result mechanically stable, hydrophobic column supports for RPC as well as IP-RP-HPLC and CEC application

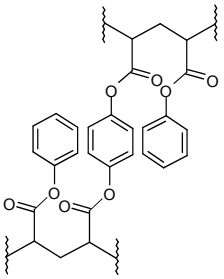


Methacrylate monoliths have been fabricated by free radical polymerisation of a number of methacrylate monomers and crosslinkers, whose combination allowed the creation of monolithic columns with different chemical properties (RP, HIC, HILIC) and functionalities (ion-exchange, IMAC, bioreactors).

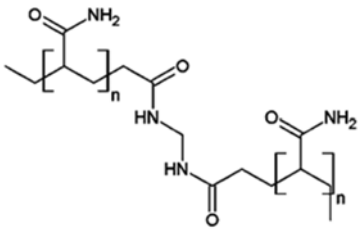


Organic Monoliths

Acrylate monoliths have been prepared by free radical polymerisation of various acrylate monomers and crosslinkers. The application of acrylate polymer columns, seem to be more focussed on CEC than HPLC.



Acrylamide monoliths as well as methacrylamide monoliths have been introduced as hydrophilic column support materials for CEC of small molecules, NPC supports for the analysis of low-molecular-weight compounds and as HIC supports for proteins.



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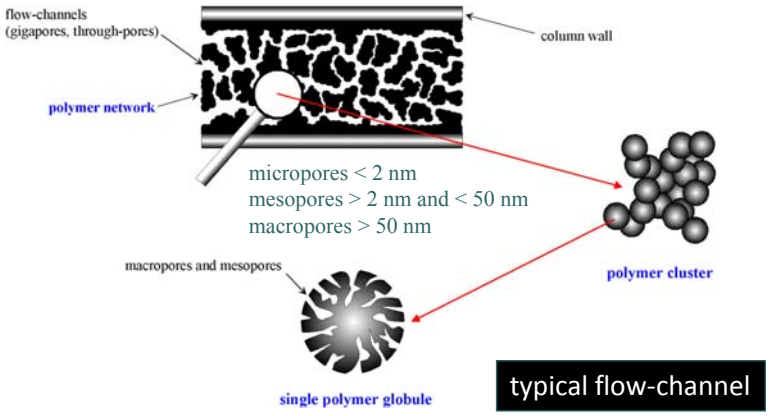
Porosity

The overall porosity of the monolithic polymer can be influenced and controlled by the nature and composition of the porogenic solvent as well as the amount of crosslinker.

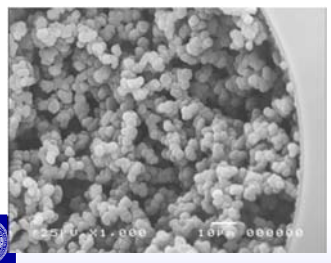
- Free space inside the polymer microglobules that precipitate at early stages of polymerisation as (monomer) swollen globules.
- Free space inside the polymer clusters, arising after chemical linkage of microglobules in solution.
- Space between the polymer clusters that build the scaffold by chemical linkage at late stages of polymerisation.

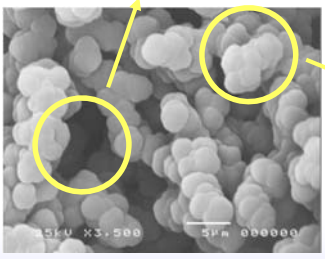
Organic Monolithic Materials

Single piece of porous polymer located inside the confines of a column



typical flow-channel



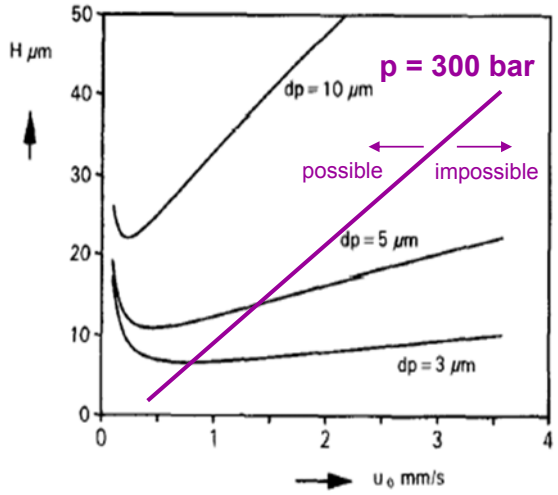


cluster of porous polymer globules

Advantages

- Reduced resistance to mass transfer due to convection
- Reduced flow resistance – high permeabilities
- Easy to prepare – no packing required
- High reproducibility, high pH stability
- No frits required
- No interparticular voids

Consequence: Possible Solution:

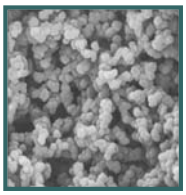


→ Pressure as the practical limit of the performance of HPLC

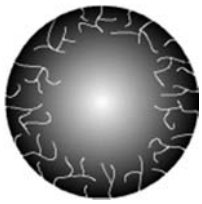
Improvement of the HPLC hardware in terms of pressure upper limits

Increase the permeability by decreasing the solvent viscosity (e.g. high temperature)

Development of novel HPLC stationary phase designs (e.g. monoliths)



Why monolithic stationary phases? Advantages in the Field of Bioanalysis



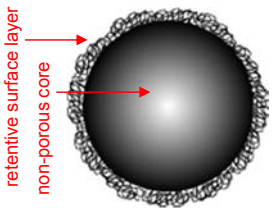
microporous particles
60 Å < pore diameter < 300 Å

Increase in average pore size

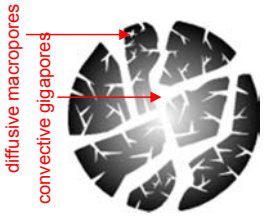
Non restricted pore diffusion of high molecular weight compounds

macroporous particles
500 Å < pore diameter < 4000 Å

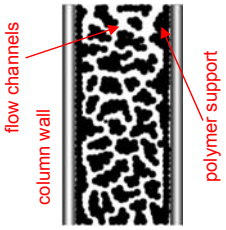
non-porous particles



micropellicular particles



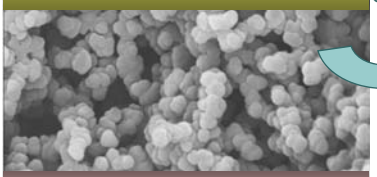
gigaporous particles



monolithic supports

Types of Monolithic Stationary Phases

Organic monoliths



styrene or (meth)acrylate building blocks

Mono-modal pore distribution of macropores

Bi-modal pore distribution of flow-channels and mesopores

Inorganic monoliths

silane building blocks

Field of application:

Separation of big molecules:
Proteomics, Genomics

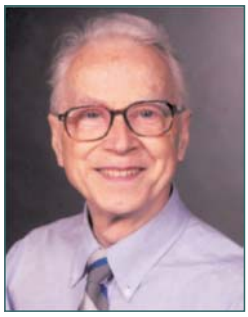
Field of application:

Separation of small molecules:
Metabolomics

→Organic and inorganic monoliths complement one another regarding applicability

Compressed Gels – Continuous Beds

Stellan Hjertén



- Hjérten introduced the concept of **compressed gels** in the late **1980s**, which have also been referred to as ***continuous beds***
- made significant contributions to a variety of areas in separation science such as electrophoresis, LC, and CEC.
- Co-polymerisation of acrylic acid and N,N'-methylene bisacrylamide resulted in highly swollen gels. Despite the high degree of compression, the gels exhibited good permeability and enabled efficient separation of proteins.

Acrylate-Based Monoliths

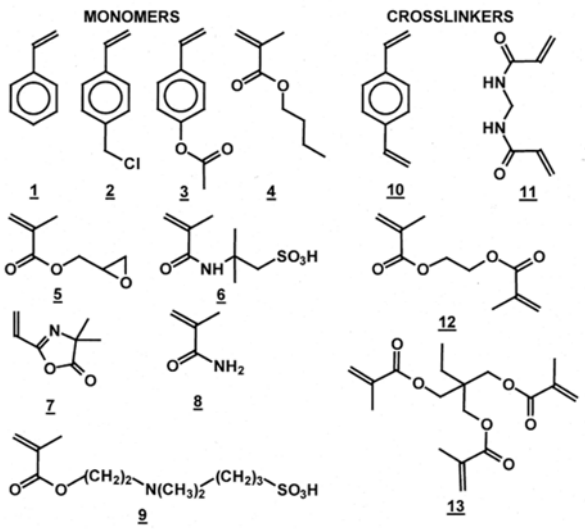
Frantisek Svec

- a pioneer in the discovery and development of porous polymeric materials and monoliths in particular.
- design of monolithic materials that are structurally and chemically optimized for various applications: LC, LC-MS, CEC, CE, microfluidic chip technology
- polymerization techniques: thermally and photochemically initiated free radical co-polymerizations, y-radiation initiated polymerizations of different organic precursors (methacrylates, styrenes,…)
- thin layers of porous polymer for thin layer separation followed by MALDI TOF MS analysis

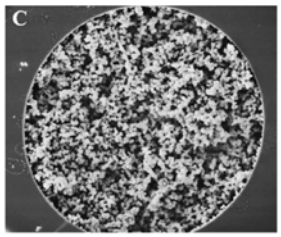
Svec, F. and Fréchet, J.M.J. Continuous rods of macroporous polymer as high-performance liquid chromatography separation media. Anal. Chem. 54, 820-822, 1992.

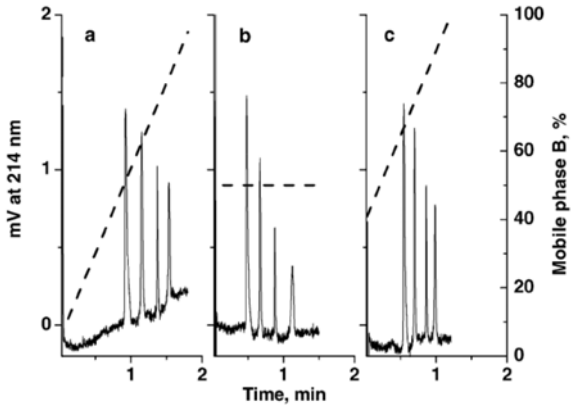
Sáfrány A., Beiler B., László K., Svec F., Control of pore formation in macroporous polymers synthesized by single-step g-radiation-initiated polymerization and cross-linking. Polymer 46, 2862-2871, 2005.

Macroporous Monoliths



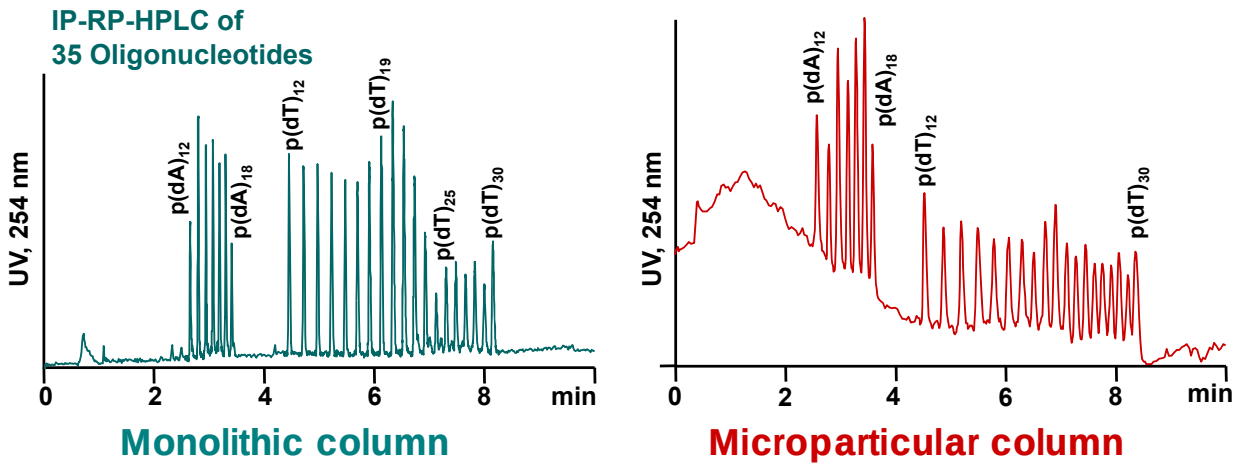
Examples of monomers used for the preparation of rigid porous polymer monolithic columns





Effect of gradient on separation of a protein mixture using monolithic column at 100 µL/min.

Comparison of Monolithic and Granular Packings

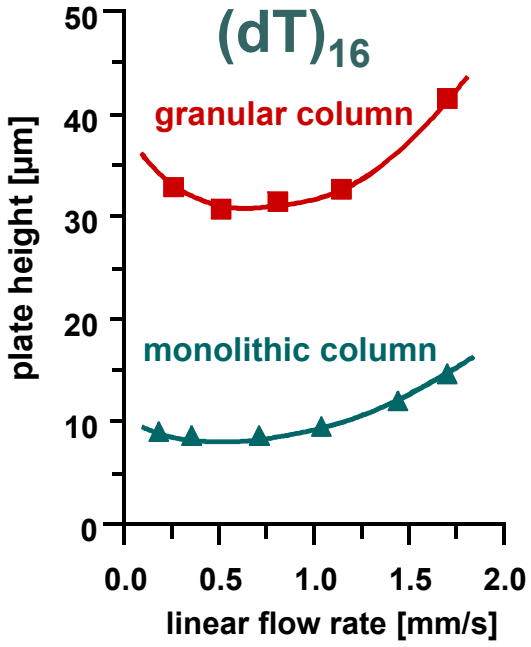


approx. 40% improvement in column efficiency

PS-DVB monolith, 60 × 0.20 mm i.d. or PS-DVB-C18, 2.1 µm, 70 × 0.20 mm i.d.; 3-9% acetonitrile in 3.5 min, 9-11% in 3.5 min, 11-11.8% (13%) in 4 min in 0.1 M triethylammonium acetate; 3.3 µL/min; 50 °C; $p(dA)_{12-18}$, $p(dT)_{12-30}$, 40-100 fmol each.

Huber et al., Anal. Chem. 71 (1999) 3730
Premstaller et al., Anal. Chem. 72 (2000) 4386.

Efficiency with Granular and Monolithic Columns



simplified van Deemter equation:

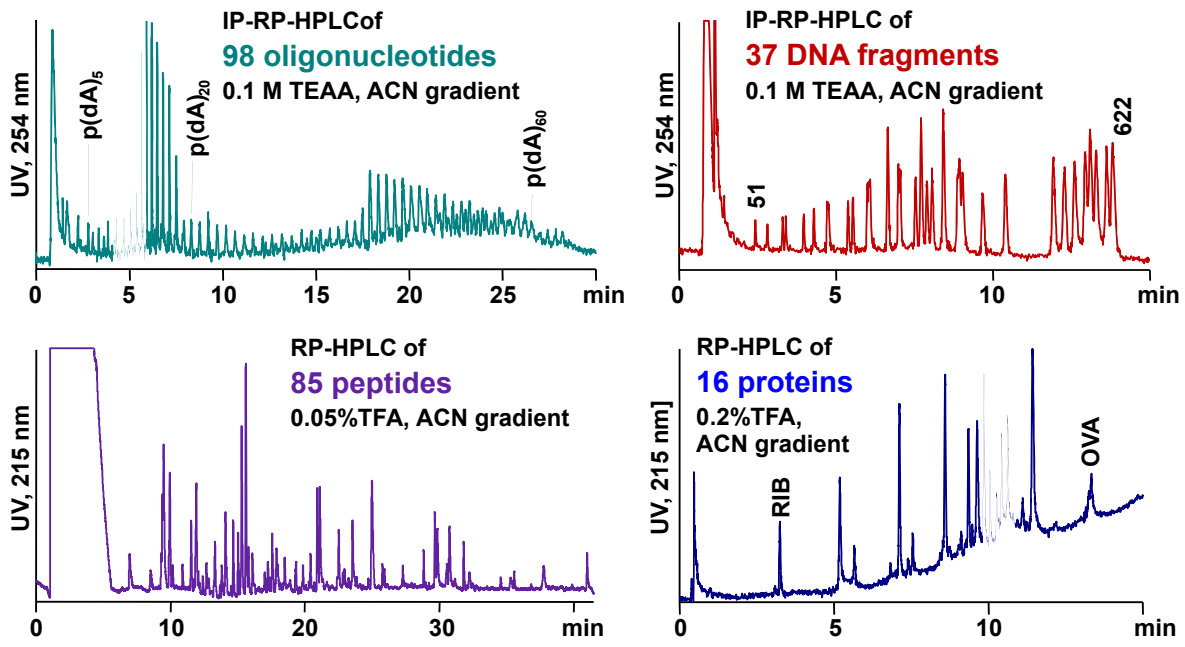
$$H = A + B/u + Cu$$

	A	B	C
granular	15.7	3.6	13.5
monolithic	3.0	0.9	6.1

60 x 0.2 mm, isocratic elution with 7.6% / 9% acetonitrile in 0.1 M triethylamminium acetate, pH 7, 50 °C, sample, $(dT)_{16}$.

Oberacher et al.,
J. Chromatogr. A 1030 (2004) 201

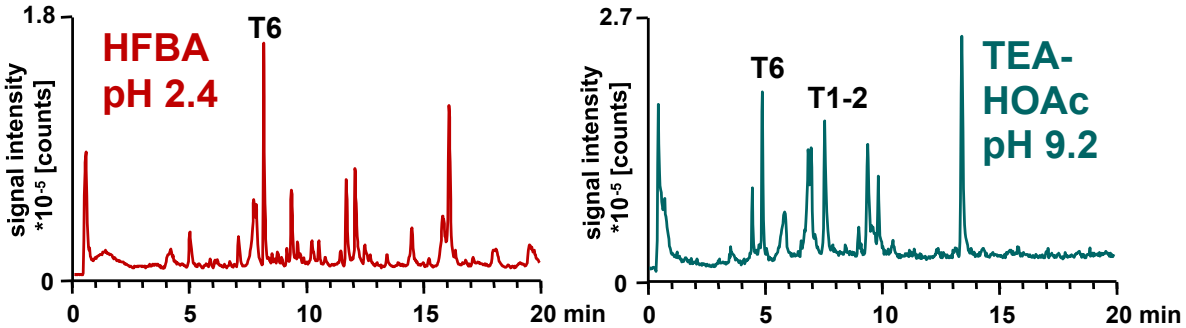
High-Resolution Bioplymer Separations



Premstaller et al., Anal. Chem. 72 (2000) 4386
Premstaller et al., Anal. Chem. 73 (2001) 2390

Institute of Analytical Chemistry and Radiochemistry, University of Innsbruck

LC-MS Analysis of β-Casein Phosphopeptides at High and Low pH



T1-2:RELEELNVPGEIVESLSSSEESITR, T6:FQSEEQQQTEDELQDK

T6: detectable in positive and negative mode ESI-MS

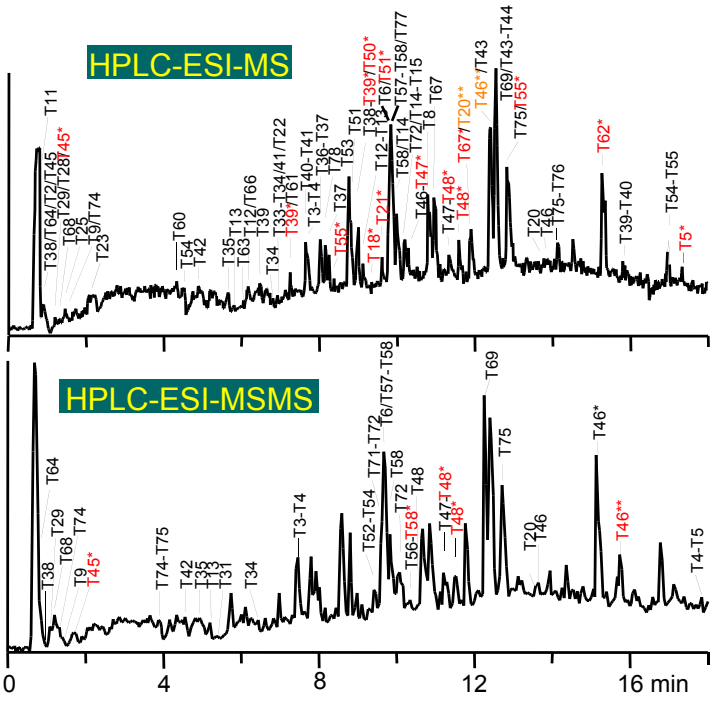
T1-2: 100 fmol undetectable in positive-, 10 fmol detectable in negative ESI-MS

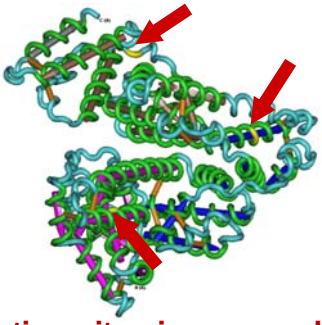
60 x 0.2 mm PS-DVB monolith, 25-min gradient of 0-37.5% acetonitrile in 0.05% heptafluorobutyric acid or 1% triethylamine-acetic acid, pH 9.2, 2 µL/min; positive or negative ESI-MS, 100 fmol β-Casein digest.

Tholey, et al., Anal. Chem., 77 (2005) 4618-4625

Institute of Analytical Chemistry and Radiochemistry, University of Innsbruck

Detection of Nitrated Peptides by LC-MS(MS)





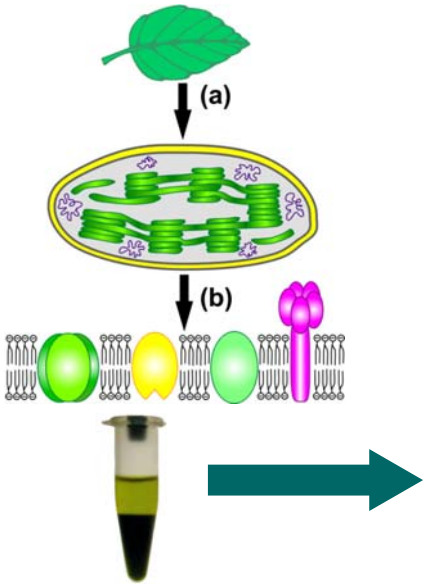
Nitration sites in serumalbumin

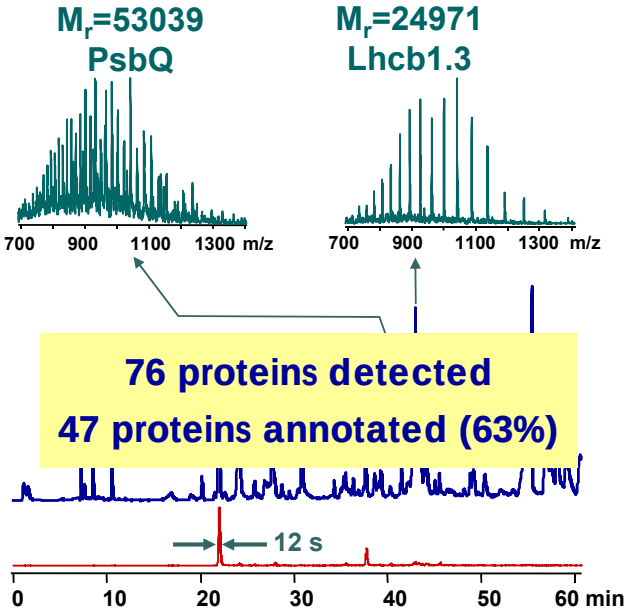
(a) Full scan MS, 500-2000 m/z
(b) Data dependent scan with dynamic exclusion, 1. full scan, 2. product ion scan of most intense precursor ion.

PS/DVB monolith, 60 x 0.20 mm i.d.; gradient, 0-40 %B acetonitrile in 0.05% aqueous trifluoroacetic acid in 20 min, 2 µl/min; 50 °C;; sample: tryptic digest of nitrated bovine serum albumin, 7.5 pmol.

Walcher et al., J. Proteome Res. 2 (2003) 534

LC-MS Analysis of Plant Proteins





PS-DVB-monolith, 60 x 0.20 mm i.d.; 5-25% acetonitrile in 0.05% trifluoroacetic acid in 10 min, 25-75 % in 80 min, 2.0 µL/min, 70 °C; 48 ng thylakoid proteins from pea.

Huber et al., Proteomics, 4 (2004) 3909, J. Biol. Chem., 276 (2001) 45755

Open Tubular Monolithic Capillary Columns

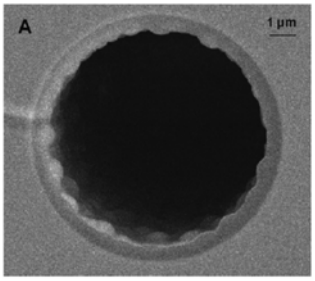
Barry L. Karger

Ultratrace LC/MS Proteomic Analysis Using 10-μm-i.d. Porous Layer Open Tubular Poly(styrene–divinylbenzene) Capillary Columns

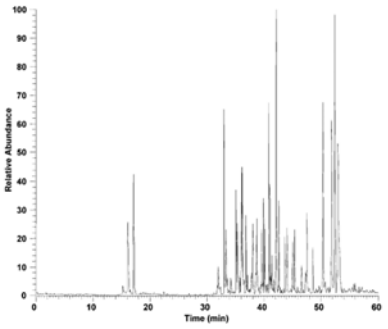
Guihua Yue,† Quanzhou Luo,† Jian Zhang,‡ Shiaw-Lin Wu, and Barry L. Karger*

Barnett Institute, Northeastern University, Boston, Massachusetts 02115

Anal. Chem. **2007**, *79*, 938–946



- Low detection levels (attomole to sub-attomole)
- peak capacity of 400
- high-resolution LC/MS at the ultratrace level.

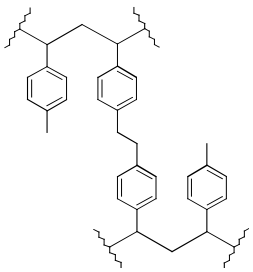
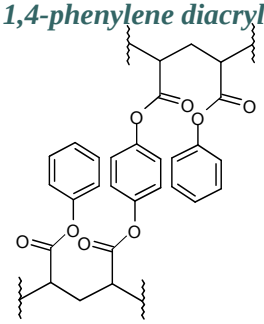
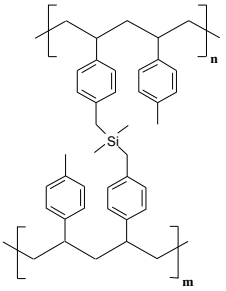


Gradient nanoLC/ESI-MS of a mixture of BSA tryptic digest and a-casein Lys-C digest. Flow-rate: ~20 nL/min.

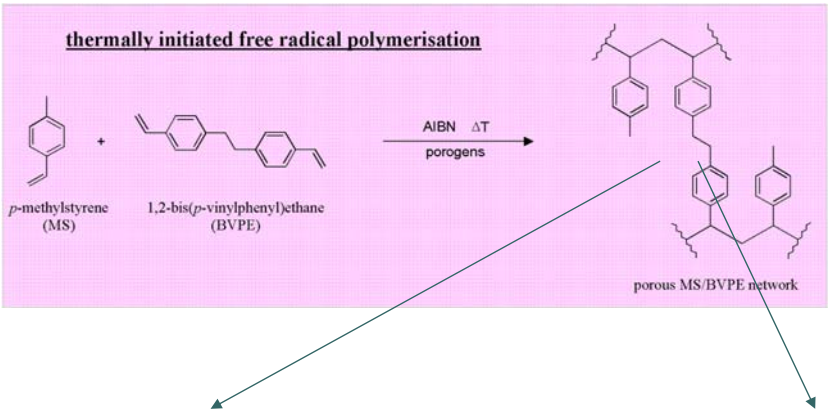
Long and high-efficiency poly(styrene-divinyl- benzene) (PS-DVB), **4.2m 10-μm-i.d. porous layer open tubular (PLOT)** capillary columns

Novel Monolithic Materials

COPOLYMERISATION OF

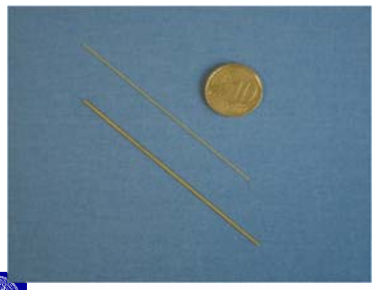
p-methylstyrene *1,2-bis(p-vinylbenzylchloride)*	*phenyl acrylate* *1,4-phenylene diacrylate*	*p-methylstyrene* *bis(p-vinylbenzyl)dimethylsilane*
		
MS/BVPE	***PA/PDA***	***MS/BVBDMS***
STYRENE RELATED STRUCTURE	*AROMATIC ACRY-LATE SYSTEM*	*INCLUDE SILANE CHEMISTRY*

MONOLITHIC MS/BVPE



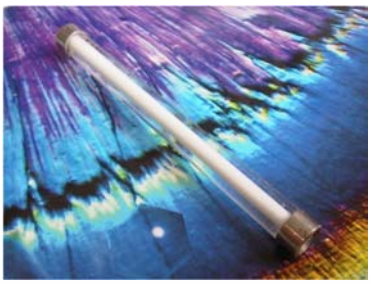
- highly crosslinked
- high crosslinking homogeneity
  due to non-conjugated crosslinker
- minimised swelling
- high pressure stability

→ **Applicable to HPLC**



capillary columns
different dimension

80 x 0.2 mm
80 x 0.533 mm



conventional HPLC columns

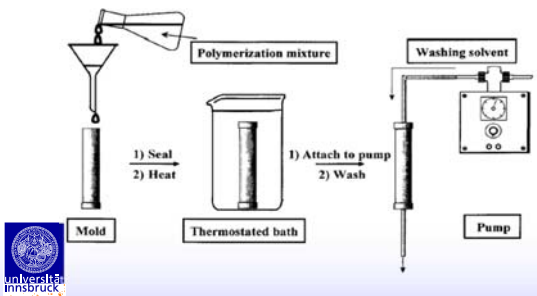
90 x 3.0 mm

General Manufacturing Process of Monoliths

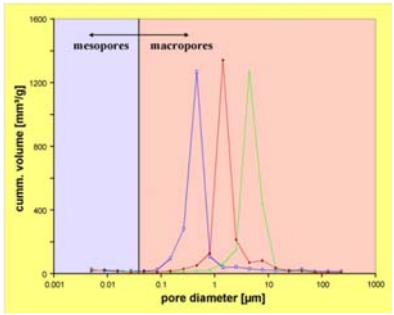
Monomer (monofunctional) + Crosslinker (bifunctional)
+
Inert diluents (porogens)
+
Initiator
Bulk polymerisation (in situ)
MONOLITH

Polymerisation parameters

- Monomer to crosslinker ratio
- Total monomers to porogen ratio
- Nature of porogens
- Nature of initiator
- Amount of initiator
- Polymerisation temperature



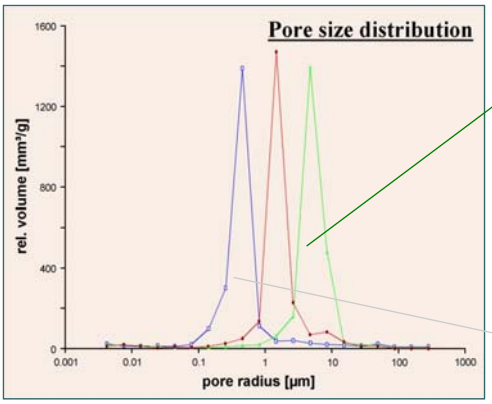
Control of the porous properties

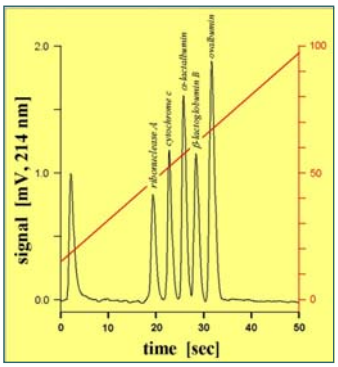


Optimisation of Monolithic MS/BVPE Capillary Columns

Control the porous properties by:

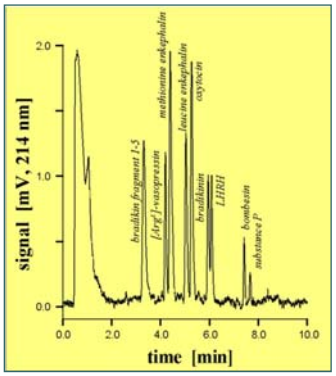
- Polymerisation temperature
- Monomer to porogen content
- Micro- to macroporogen content
- Initiator content





Swift protein separations

Efficiency towards small biomolecules e.g. peptides, oligonucleotides

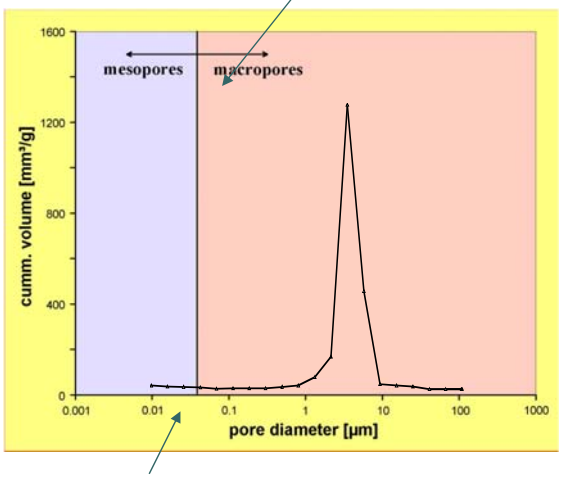


→AIM: Combination of high flow characteristics with separation efficiency

Influence of Porosity

Usual polymerisation parameters

Flow channels get too narrow

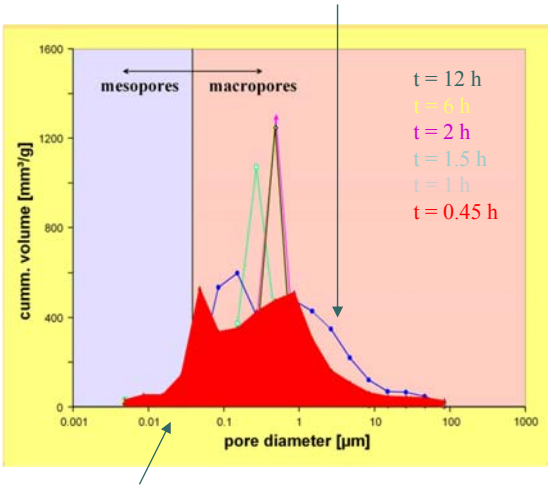


Shift in mesopore range

→ **Zero permeable support**

Polymerisation time as novel polymerisation parameter

Fraction of flow channels still high



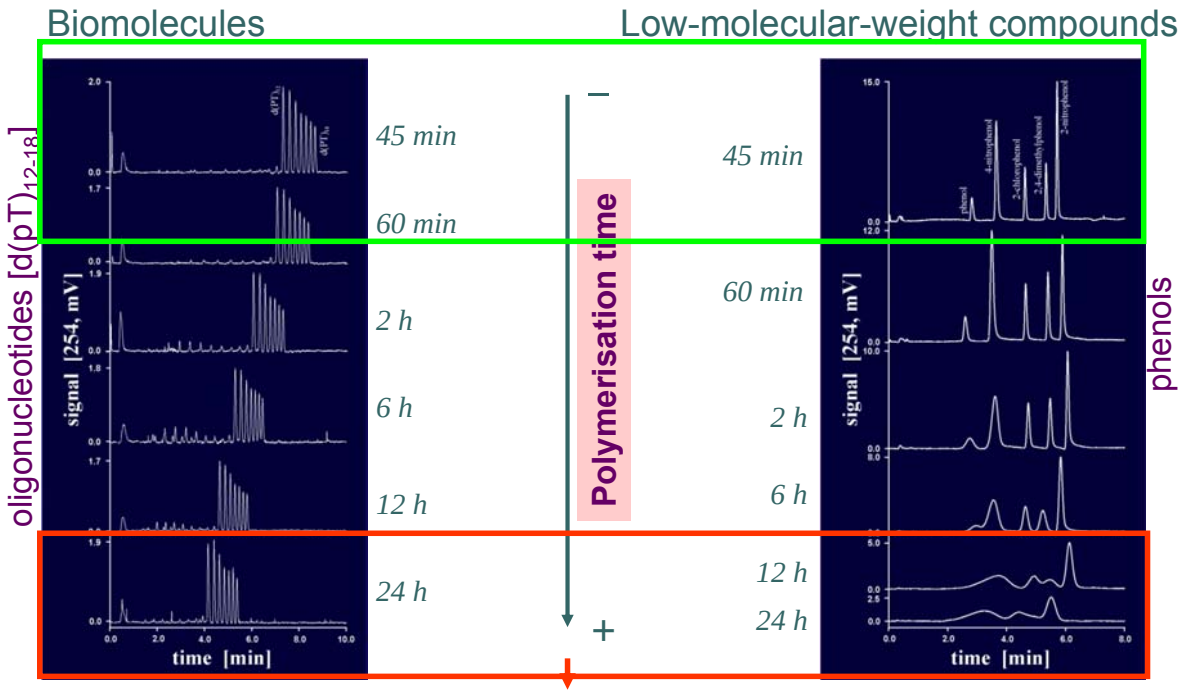
Increase in fraction of mesopores

→ **Highly permeable support with mesopores**

L. Trojer, G.K. Bonn *Monolithic organic copolymer*, 2007, US Appl. Number: 11/551,181

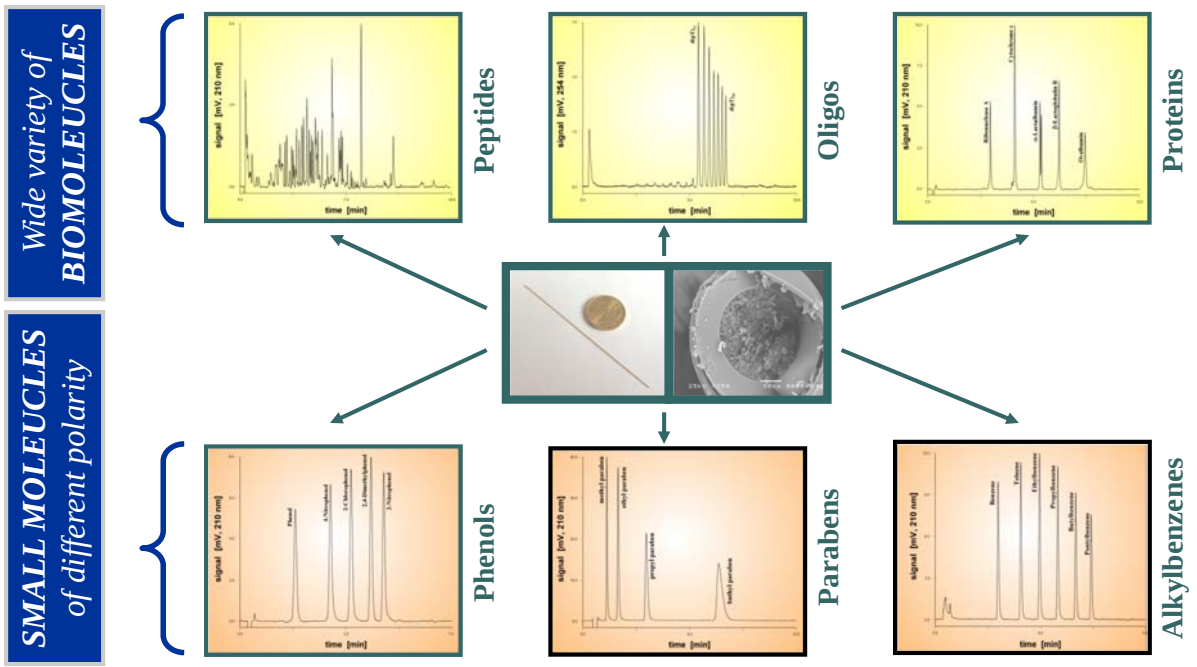
Monolithic Separation

Impact on the Separation Efficiency of Monolithic MS/BVPE



Achievable also with commercial monolithic organic capillary columns

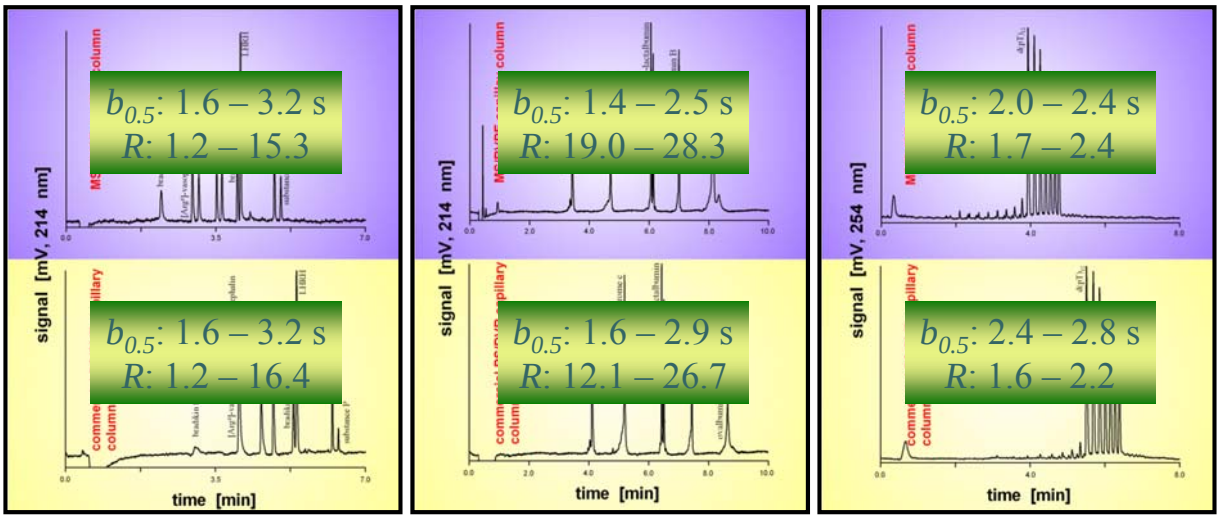
Extended Applicability of the shortly Polymerised Monoliths



→ Separation of biopolymers as well as small molecules of various polarity can be separated on the same monolithic capillary column

Comparison to a Commercial Monolithic Styrene Support

1) Separation efficiency in comparison

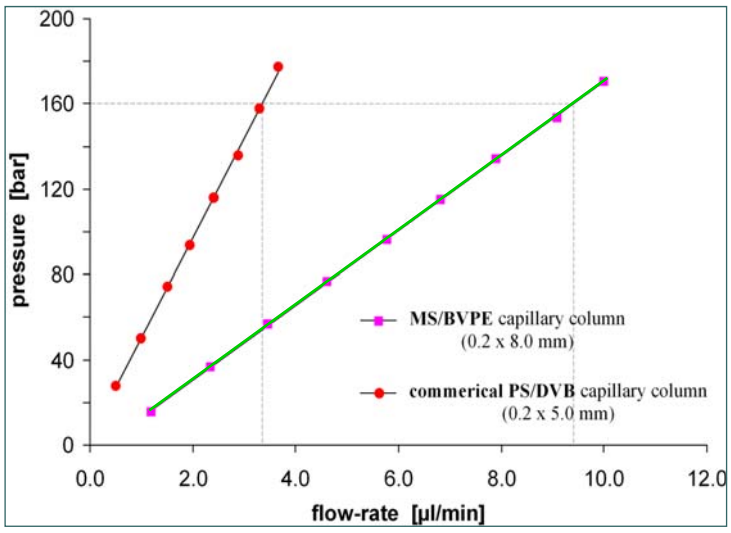


PEPTIDES **PROTEINS** **OLIGOS**

→ Chromatographic performance comparable to commercial „state of the art“ styrene monoliths

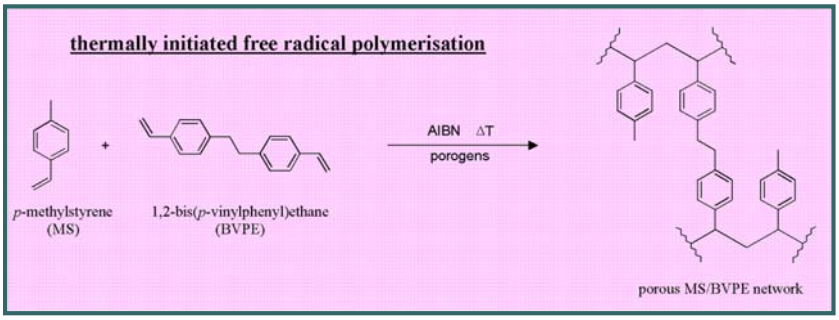
Comparison to a Commercial Monolithic Styrene Support

2) Permeability in comparison

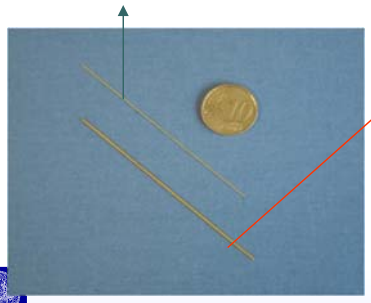


→ Separation efficiency comparable to commercially available supports, but high permeability enables high speed separations

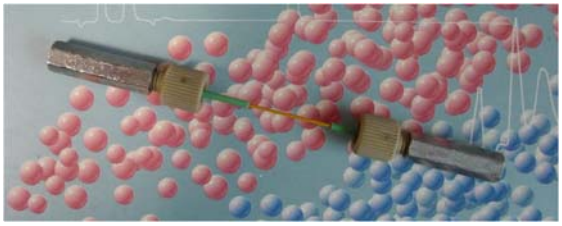
Monolithic MS/BVPE Capillary Column with Extended Dimension: FABRICATION OF 0.533 mm I.D. COLUMNS



200 µm I.D.



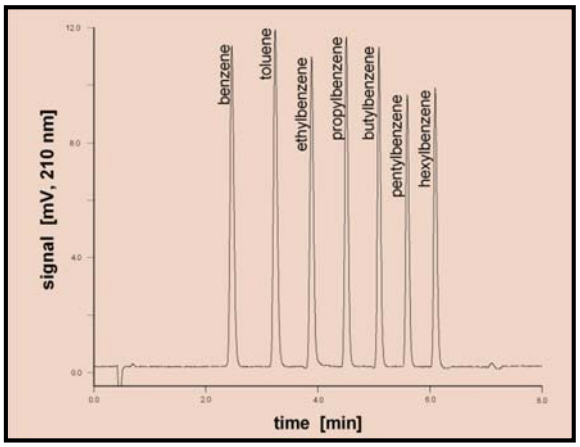
533 µm I.D.



Downscale factor of ~ 7 by reducing from 533 to 200 µm I.D. →flow-rate of 533 capillary in the range of 50-100 µl/min

Examples of Separation Efficiency of 0.533 mm I.D. Columns

Gradient separations of alkylbenzenes



analyte	t_R [min]	$b_{0.5}$ [min]	$b_{0.5}$ [sec]	R
benzene	2,472	0,07667	4,600	5,752
toluene	3,253	0,07500	4,500	4,932
ethylbenzene	3,908	0,07333	4,400	5,003
propylbenzene	4,535	0,06667	4,000	4,980
butylbenzene	5,122	0,06500	3,900	4,382
pentylbenzene	5,632	0,06500	3,900	4,322
hexylbenzene	6,135	0,06500	3,900	

Chromatographic conditions:

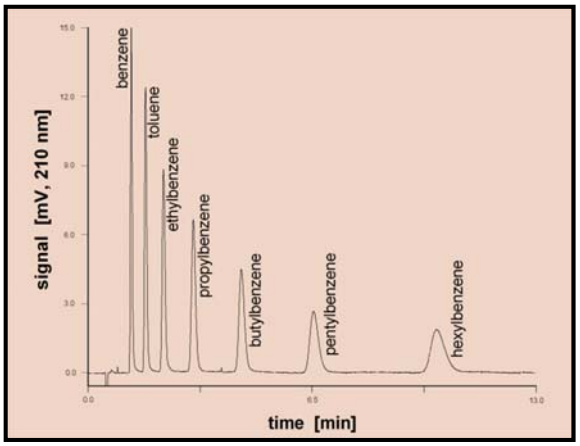
Column: MS/BVPE, 75 x 0.533 mm I.D.,
Solvents: A: water, B: ACN,
Gradient: 40 % -100 % B in 5 min and 100 % B for 1 min,
Flow rate: 50 µl/min,
Pressure: 120 bar (@ 40 % B),
Temperature: RT,
Detection: UV 210 nm,
Injection.: 500 nl, 12.5 ng each alkylbenzene

Alkylbenzene sample dissolved in 60 % ACN

Column prepared by a total Polymerisation time of 90 min at 65 °C

Examples of Separation Efficiency of 0.533 mm I.D. Columns

Isocratic separations of alkylbenzenes



Chromatographic conditions:

Column: MS/BVPE, 75 x 0.533 mm I.D.,
Solvents: A: water, B: ACN,
Conditions: isocratic at 65 % B,
Flow rate: 50 µl/min,
Pressure: 95 bar (@ 65 % B),
Temperature: RT,
Detection: UV 210 nm,
Injection.: 500 nl, 12.5 ng each alkylbenzene

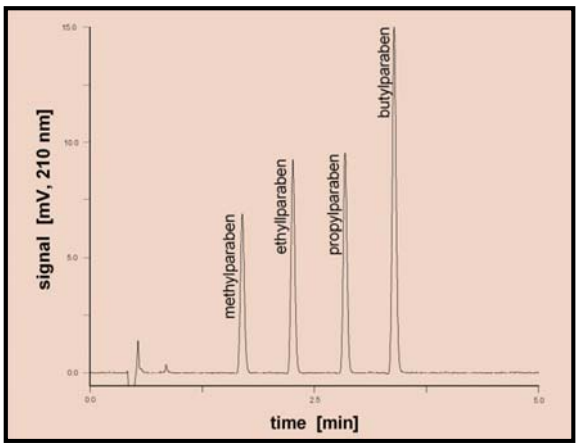
Alkylbenzene sample dissolved in 60 % ACN

analyte	t_R [min]	$b_{0.5}$ [min]	$b_{0.5}$ [sec]	N/meter	H [µm]
benzene	1,245	0,05333	3,200	37741	26,50
toluene	1,660	0,07167	4,300	37150	26,92
ethylbenzene	2,182	0,09167	5,500	39235	25,49
propylbenzene	3,048	0,12833	7,700	39065	25,60
butylbenzene	4,448	0,17667	10,600	43896	22,78
pentylbenzene	6,548	0,27500	16,500	39262	25,47
hexylbenzene	10,120	0,44167	26,500	36357	27,51

Column prepared by a total polymerisation time of 90 min at 65 °C

Examples of Separation Efficiency of 0.533 mm I.D. Columns

Gradient separations of parabens



Chromatographic conditions:

Column: MS/BVPE, 75 x 0.533 mm I.D.,
Solvents: A: 0.1 % TFA, B: ACN/0.1 % TFA,
Gradient: 20 % -90 % B in 5 min and
90 % B for 1 min,
Flow rate: 50 µl/min,
Pressure: 130 bar (@ 20 % B),
Temperature: RT,
Detection: UV 210 nm,
Injection.: 500 nl, 7.5 - 18 ng each paraben

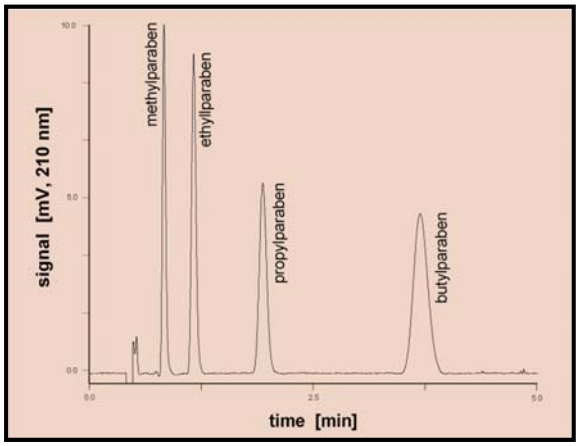
Paraben sample dissolved in 60 % MeOH

analyte	t_R [min]	$b_{0.5}$ [min]	$b_{0.5}$ [sec]	R
methylparaben	1,697	0,05000	3,000	6,396
ethylparaben	2,260	0,04833	2,900	6,726
propylparaben	2,842	0,04833	2,900	6,202
butylparaben	3,388	0,05000	3,000	

Column prepared by a total Polymerisation time of 90 min at 65 °C

Examples of Separation Efficiency of 0.533 mm I.D. Columns

Isocratic separations of parabens



Chromatographic conditions:

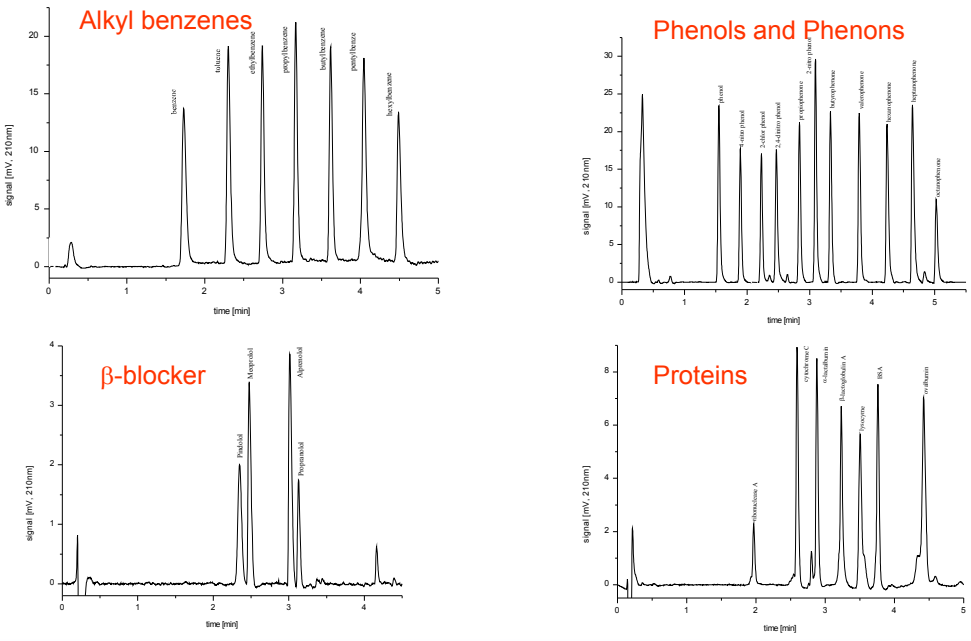
Column: MS/BVPE, 75 x 0.533 mm I.D.,
Solvents: A: 0.1 % TFA, B: ACN/0.1 % TFA,
Conditions: isocratic at 35 % B,
Flow rate: 50 µl/min,
Pressure: 130 bar (@ 35 % B),
Temperature: RT,
Detection: UV 210 nm,
Injection.: 500 nl, 7.5 - 18 ng each paraben

Paraben sample dissolved in 60 % MeOH

analyte	t_R [min]	$b_{0.5}$ [min]	$b_{0.5}$ [sec]	N/meter	H [µm]
methylparaben	0,837	0,04012	2,407	30140	33,18
ethylparaben	1,168	0,05167	3,100	35386	28,26
propylparaben	1,942	0,09110	5,466	31469	31,78
butylparaben	3,703	0,17880	10,728	29702	33,67

Column prepared by a total polymerisation time of 90 min at 65 °C

Separation – BVPE - Monoliths



High-Resolution Separation of dsDNA Fragments

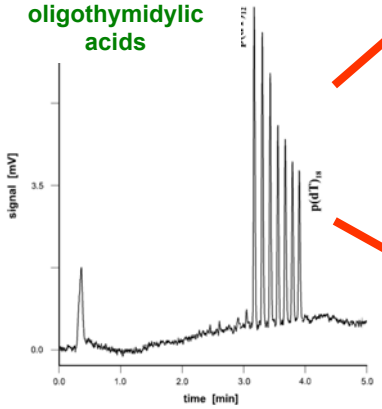
peak width at half peak height:

1.6 – 1.8 sec.

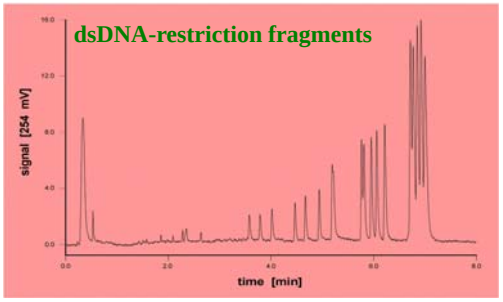
while still having high resolution:

2.6-2.1

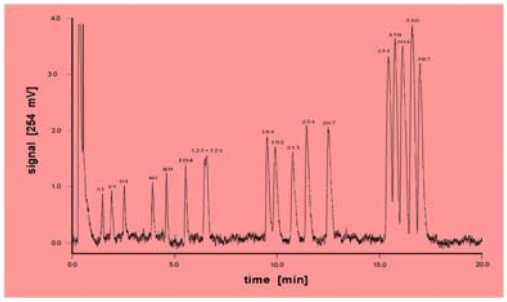
oligothymidylic acids



solvent A: 0.1 M TEAA, pH 7, solvent B: 0.1 M TEAA in 40 % ACN, pH 7, 0-20 % B in 1 min and 20-40 % B in 7, 80x0.2 mm I.D., 8 µl/min, 60 °C, UV 254, sample: $p(dT)_{12-18}$, ~ 18 fmol each



solvent A: 0.1 M TEAA, pH 7, solvent B: 0.1 M TEAA in 40 % ACN, pH 7, 0-20 % B in 1 min and 20-60 % B in 10 min, 80x0.2 mm I.D., 7 µl/min, 50 °C, UV 254, sample: pBR322 /HaeIII, ~25 ng total



solvent A: 0.1 M TEAA, pH 7, solvent B: 0.1 M TEAA in 40 % ACN, pH 7, 17-25 % B in 5 min and 25-35 % B in 11 min, 80x0.2 mm I.D., 7.5 µl/min, 50 °C, UV 254, sample: pBR322 /HaeIII, ~25 ng total

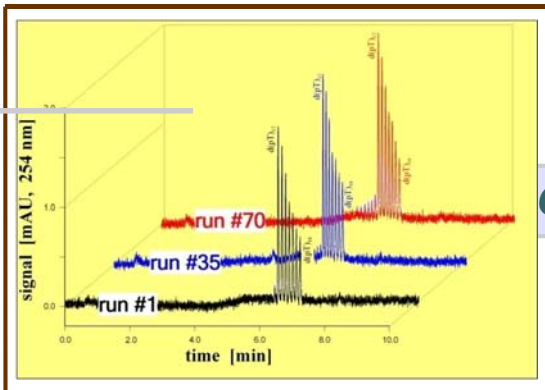
Novel Monolithic Capillary Columns

REPRODUCIBILITY STUDIES

Institute of Analytical Chemistry and Radiochemistry, University of Innsbruck

Run-to-Run and Long-term Stability

RSD:
< 2 % for t_R
< 7 % for $b_{0.5}$
< 8 % for R



analyte	t_R [min]	$b_{0.5}$ [s]	R
$d(pT)_{12}$	6.685	1.488	2.297
$d(pT)_{13}$	6.795	1.722	2.116
$d(pT)_{14}$	6.903	1.698	2.094
$d(pT)_{15}$	7.008	1.662	2.125
$d(pT)_{16}$	7.113	1.650	1.959
$d(pT)_{17}$	7.217	1.908	1.979
$d(pT)_{18}$	7.332	1.986	
separation within	0.647		

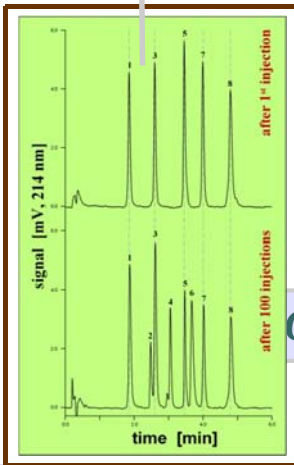
Injection 1

analyte	t_R [min]	$b_{0.5}$ [s]	R
$d(pT)_{12}$	6.462	1.410	2.104
$d(pT)_{13}$	6.553	1.488	2.118
$d(pT)_{14}$	6.648	1.518	2.106
$d(pT)_{15}$	6.747	1.632	2.036
$d(pT)_{16}$	6.848	1.692	2.015
$d(pT)_{17}$	6.953	1.800	1.874
$d(pT)_{18}$	7.057	1.920	
separation within	0.595		

Injection 35

analyte	t_R [min]	$b_{0.5}$ [s]	R
$d(pT)_{12}$	6.562	1.590	2.370
$d(pT)_{13}$	6.677	1.662	2.266
$d(pT)_{14}$	6.790	1.680	2.242
$d(pT)_{15}$	6.903	1.698	2.093
$d(pT)_{16}$	7.010	1.728	2.178
$d(pT)_{17}$	7.127	1.872	1.960
$d(pT)_{18}$	7.237	1.890	
separation within	0.675		

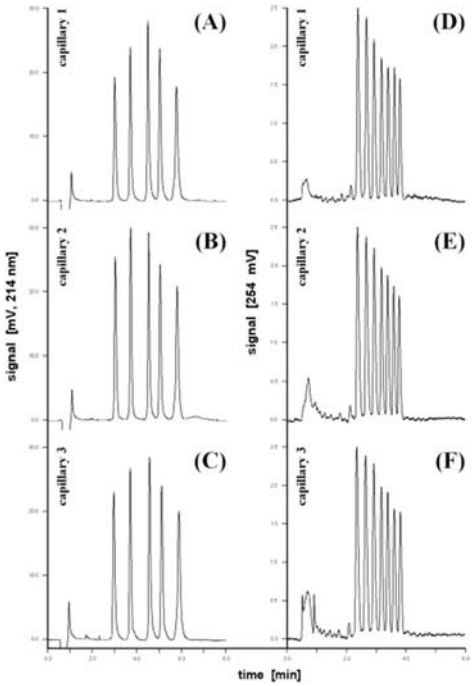
Injection 70



Peak number	Protein	t_R [min]	$b_{0.5}$ [s]	R	t_R [min]	$b_{0.5}$ [s]	R
		Run # 1			Run # 100		
1	ribonuclease A	1.85	3.80	6.90	1.87	4.10	6.30
2	insulin				2.48	3.20	
3	cytochrome *c*	2.59	3.80	8.18	2.61	4.20	7.23
4	lysozyme				3.05	3.30	
5	α-lactalbumin	3.45	3.60	5.15	3.42	3.70	5.56
6	BSA				3.67	4.90	
7	β-lactoglobulin B	3.99	3.80	6.28	4.02	3.90	6.06
8	ovalbumin	4.79	5.20	--	4.81	5.30	--

→ Stable for at least 100 injections, high run-to-run reproducibility

Batch-to-Batch Reproducibility

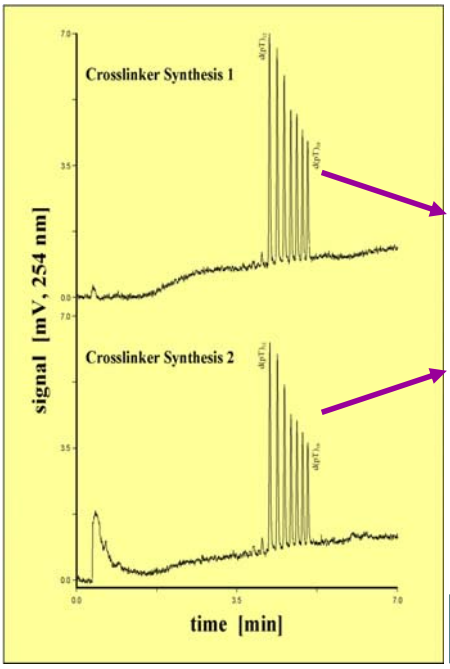


Analyte	Capillary 1			Capillary 2			Capillary 3			Standard deviation [%]		
	t_R [min]	$b_{0.5}$ [s]	R	t_R [min]	$b_{0.5}$ [s]	R	t_R [min]	$b_{0.5}$ [s]	R	t_R	$b_{0.5}$	R
Proteins												
ribonuclease A	2.99	5.73	4.37	3.02	5.53	4.61	2.96	5.83	4.65	1.14	2.68	3.38
cytochrome *c*	3.67	5.30	5.53	3.72	5.07	5.78	3.69	5.30	5.78	0.60	2.58	2.68
α-lactalbumin	4.48	5.07	3.36	4.52	4.80	3.52	4.54	5.07	3.42	0.64	3.09	2.27
β-lactoglobulin B	5.01	5.90	4.14	5.04	5.57	4.34	5.07	5.97	4.08	0.67	3.69	3.23
ovalbumin	5.77	7.13	--	5.81	7.03	--	5.83	7.17	--	0.55	0.98	--
Oligonucleotides												
$d(pT)_{12}$	2.35	5.11	2.01	2.30	5.30	1.90	2.33	5.60	1.92	1.05	4.72	2.94
$d(pT)_{13}$	2.64	5.13	1.82	2.59	5.50	1.83	2.63	5.50	1.88	0.98	3.94	1.81
$d(pT)_{14}$	2.90	5.07	1.82	2.87	5.00	1.84	2.92	5.33	1.83	0.96	3.44	0.65
$d(pT)_{15}$	3.16	4.83	1.69	3.12	4.67	1.62	3.17	5.00	1.63	1.10	3.45	2.32
$d(pT)_{16}$	3.39	4.67	1.67	3.33	4.60	1.62	3.41	4.87	1.59	1.26	2.95	2.60
$d(pT)_{17}$	3.61	4.53	1.49	3.54	4.50	1.47	3.63	4.87	1.48	1.36	4.38	1.05
$d(pT)_{18}$	3.80	4.53	--	3.72	4.40	--	3.83	4.70	--	1.44	3.31	--

RSD:
< 2 % for t_R
< 5 % for $b_{0.5}$
< 4 % for R

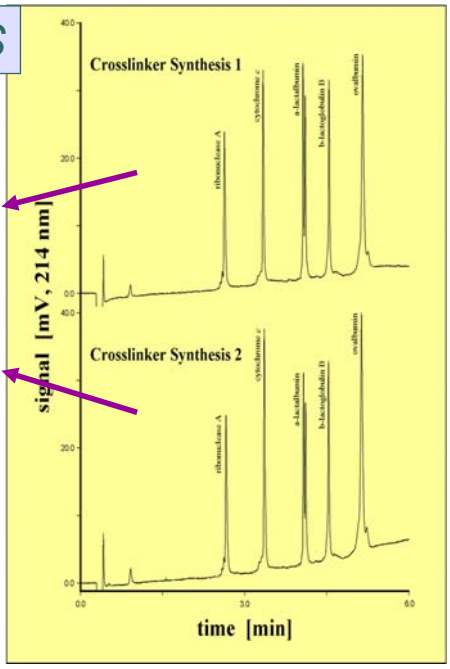
→High reproducibility of monolith fabrication is given

Reproducibility of the Crosslinker Synthesis



PROTEINS

RSD:
< 1 % for t_R
< 4 % for $b_{0.5}$
< 8 % for R



OLIGOS

Summary

RSD:
< 2 % for t_R
< 5 % for $b_{0.5}$
< 4 % for R

RUN-TO-RUN REPRODUCIBILITY

Longterm stability, stability of the support under permanent pressure conditions

RSD:
< 2 % for t_R
< 7 % for $b_{0.5}$
< 8 % for R

BATCH-TO-BATCH REPRODUCIBILITY

Fabrication of the monolithic capillaries: comprises preparation of the polymerisation mixtures, filling into the capillaries and polymerisation

RSD:
< 1 % for t_R
< 4 % for $b_{0.5}$
< 8 % for R

CROSSLINKER REPRODUCIBILITY

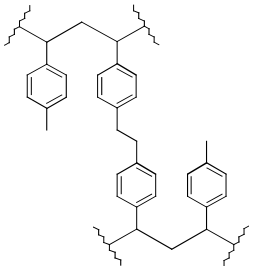
Synthesis and purification of the crosslinker

→Reproducibility in all cases in the same order of magnitude than the precision of a typical µ-HPLC system

NOVEL MONOLITHIC MATERIALS

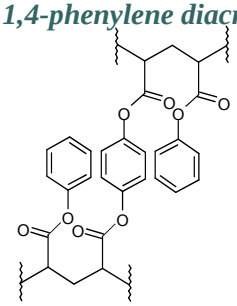
COPOLYMERISATION OF

p-methylstyrene
1,2-bis(p-vinylbenzylchloride)



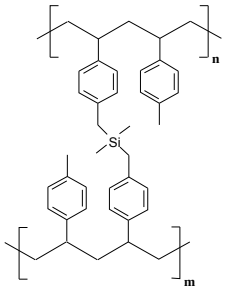
MS/BVPE

phenyl acrylate
1,4-phenylene diacrylate



PA/PDA

p-methylstyrene
bis(p-vinylbenzyl)dimethylsilane



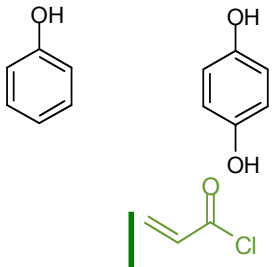
MS/BVBDMS

STYRENE RELATED STRUCTURE

AROMATIC ACRY-LATE SYSTEM

INCLUDE SILANE CHEMISTRY

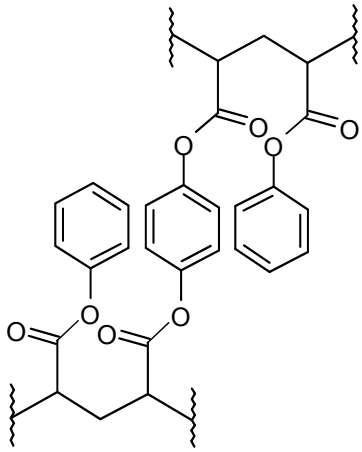
MONOLITHIC PA/PDA



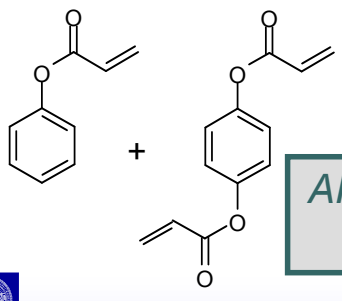
AROMATIC EDUCTS

Et_3N in THF
RT

Porogens, AIBN
DT



MONOLITHIC AROMATIC ACRYLATE COPOLYMER

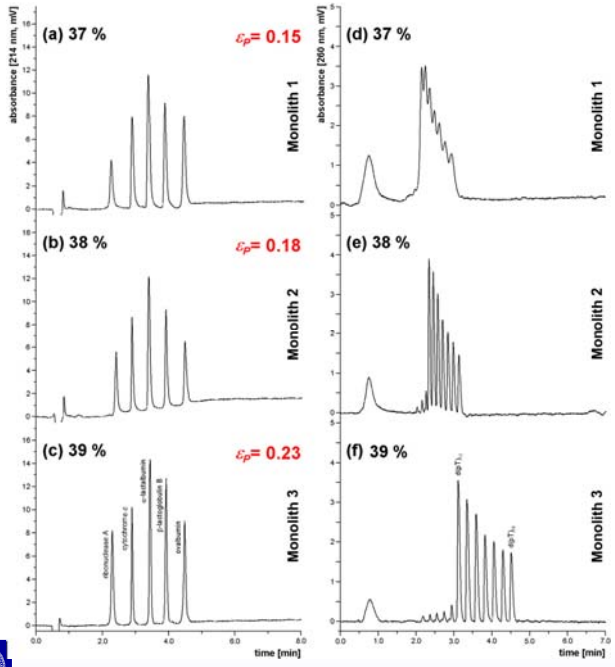


AROMATIC ACRYLATE PRECURSORS

G.K. Bonn, S. Lubbad, C.P. Bisjak, *Monolithic Organic Copolymer*, 2007, US-Patent pending

Effect of Monomer Content on Biopolymer Separation

OLIGONUCLEOTIDES



Retention behaviour (t_R) of PROTEINS comparable on all Monoliths, but peak sharpness and thus chromatographic efficiency is improved upon increasing the monomer/porogen ratio.

t_R and chromatographic efficiency of OLIGONUCLEOTIDES is much more affected by porosity changes.

OLIGONUCLEOTIDES (small analytes) more sensitive to porosity changes than PROTEINS (large analytes).

Generally, the higher e_P (volume fraction of pores), the better the chromatographic efficiency for both, large and small biomolecules.

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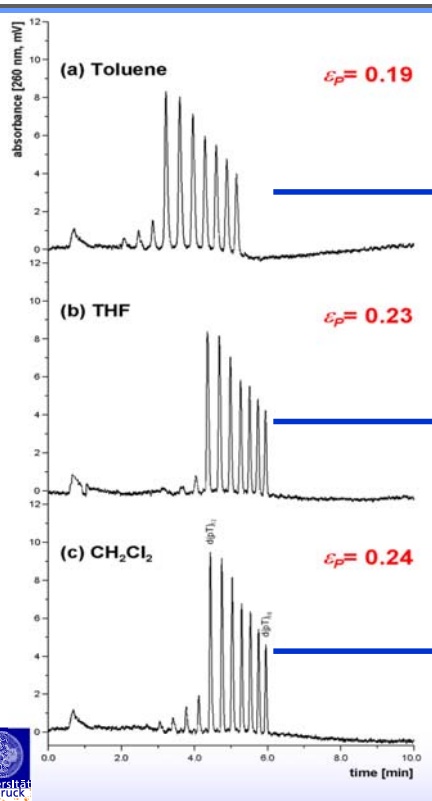
Effect of Microporogen Nature on Biopolymer Separation



Compound	Toluene		
	t_R [min]	$b_{0.5}$ [min]	R
$d(pT)_{12}$	3.10	0.09	2.60
$d(pT)_{13}$	3.48	0.09	2.49
$d(pT)_{14}$	3.84	0.09	2.33
$d(pT)_{15}$	4.17	0.08	2.05
$d(pT)_{16}$	4.47	0.09	1.90
$d(pT)_{17}$	4.76	0.09	1.77
$d(pT)_{18}$	5.03	0.09	-
	THF		
	t_R [min]	$b_{0.5}$ [min]	R
$d(pT)_{12}$	4.34	0.07	2.90
$d(pT)_{13}$	4.67	0.07	2.64
$d(pT)_{14}$	4.97	0.07	2.54
$d(pT)_{15}$	5.25	0.06	2.24
$d(pT)_{16}$	5.49	0.06	2.15
$d(pT)_{17}$	5.72	0.06	1.96
$d(pT)_{18}$	5.93	0.06	-
	CH_2Cl_2		
	t_R [min]	$b_{0.5}$ [min]	R
$d(pT)_{12}$	4.43	0.06	3.08
$d(pT)_{13}$	4.75	0.06	2.78
$d(pT)_{14}$	5.03	0.06	2.71
$d(pT)_{15}$	5.29	0.05	2.54
$d(pT)_{16}$	5.53	0.06	2.30
$d(pT)_{17}$	5.75	0.06	2.14
$d(pT)_{18}$	5.95	0.05	-

Changing the MICRO-POROGEN NATURE enables the fabrication of monoliths possessing different porous properties without changing the composition of the polymerisation mixture.

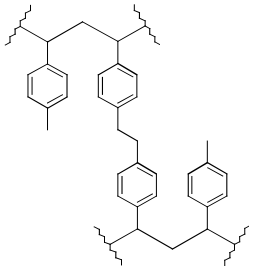
Regarding the separation of OLIGONUCLEOTIDES, t_R, $b_{0.5}$ and hence R are strongly influenced by the nature of the microporogen that is used for monolith synthesis.

Institute of Analytical Chemistry and Radiochemistry, University of Innsbruck

NOVEL MONOLITHIC MATERIALS

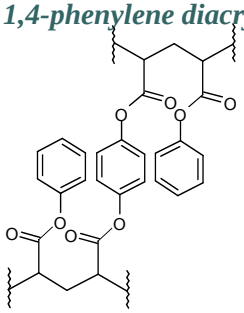
COPOLYMERISATION OF

p-methylstyrene
1,2-bis(p-vinylbenzylchloride)



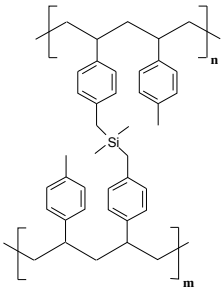
MS/BVPE

phenyl acrylate
1,4-phenylene diacrylate



PA/PDA

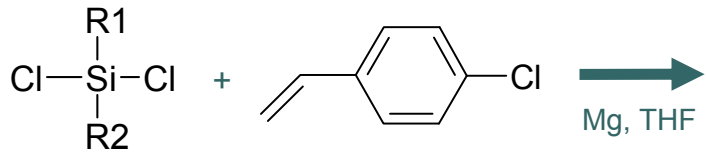
p-methylstyrene
bis(p-vinylbenzyl)dimethylsilane

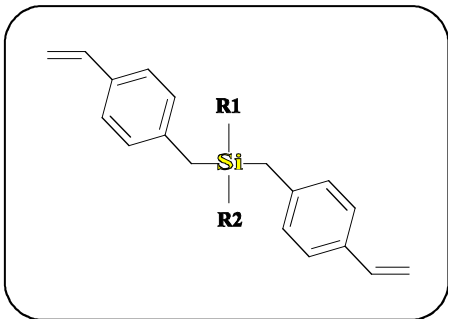


MS/BVBDMS

STYRENE RELATED STRUCTURE	AROMATIC ACRY-LATE SYSTEM	INCLUDE SILANE CHEMISTRY

MONOLITHIC MS/BVBDMS

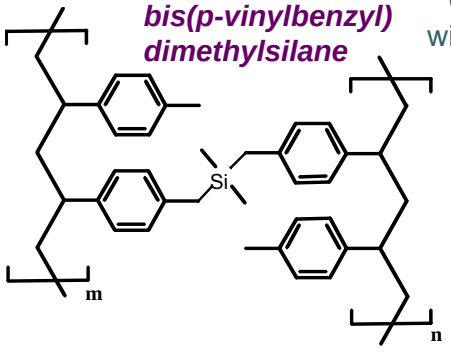


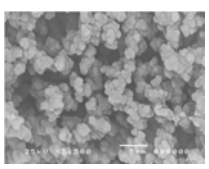


Silane Crosslinker

p-methylstyrene
bis(p-vinylbenzyl)
dimethylsilane

Copolymerisation
with *p*-methylstyrene

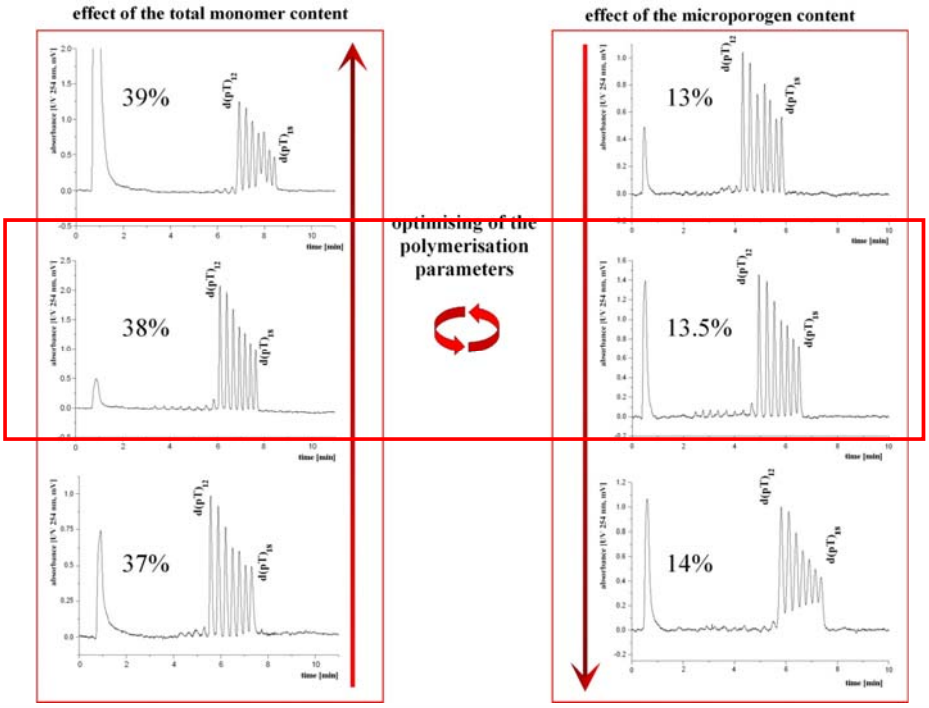




R1:	R2:
CH3	CH3
CH3	Phenyl
CH3	H
CH3	C8
CH3	C18

Easy access to different functionalities and monolithic materials with different properties

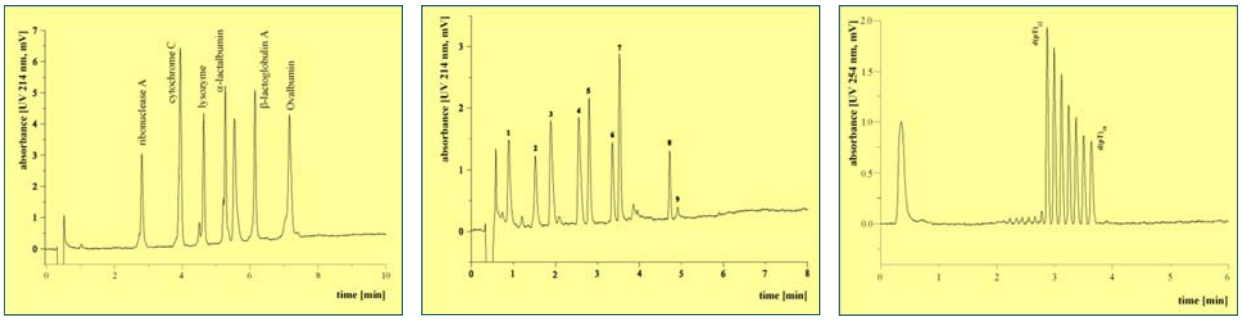
Effect Regarding Chromatographic Performance



Optimised mixture: 38% (w/w) total monomer content; 13.5% (w/w) microporogen content

Separation Examples on Optimised MS/BVBDMS

PROTEINS	*PEPTIDES*	*OLIGOS*



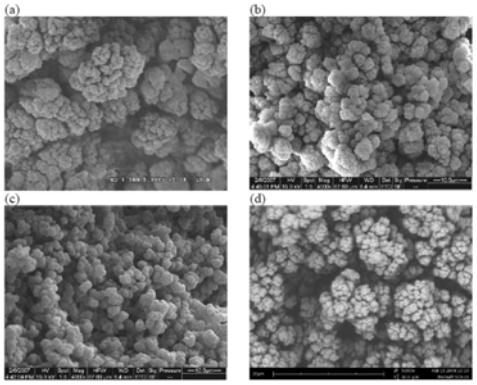
Chromatographic parameters:

$3.2\ s < b_{0.5} < 5.2\ s$ $2.3 < R < 10.0$	$2.8\ s < b_{0.5} < 4.8\ s$ $1.8 < R < 14.0$	$2.3\ s < b_{0.5} < 3.8\ s$ $1.7 < R < 1.8$

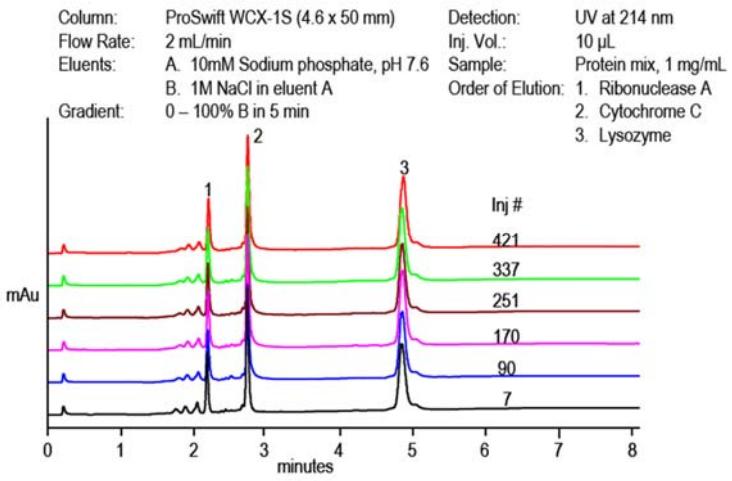
→ High efficiency towards a wide variety of biomolecules

Ion Exchange Monolithic Columns from Dionex for Biomolecule Analysis

SEM Pictures



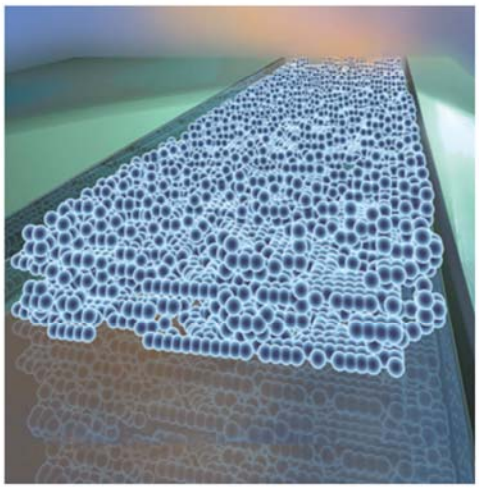
(a) ProSwift WAX-1S (5000x),
(b) ProSwift SAX-1S (4000x),
(c) ProSwift WCX-1S (4000x),
(d) ProSwift SCX-1S (5000x)

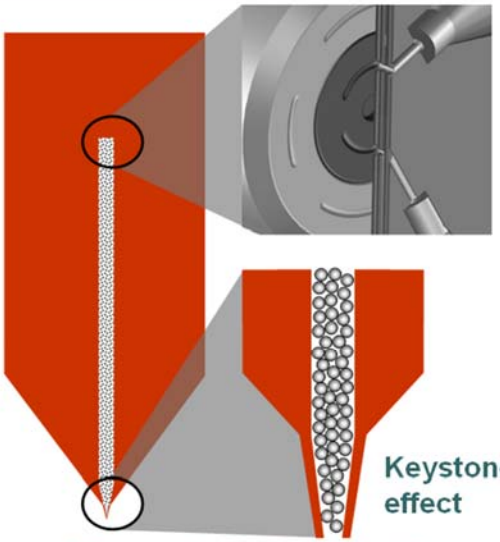


Ion Exchange Monolithic Columns from Dionex for Biomolecule Analysis

Parameter	ProSwift WAX-1S		ProSwift WCX-1S		ProSwift SAX-1S		ProSwift SCX-1S
Column dimension	4.6x50 mm	1x50 mm	4.6x50 mm	1x50 mm	4.6x50 mm	1x50 mm	4.6x50 mm
Base Matrix Material	Polymethacrylate						
Surface chemistry	Tertiary amine		Carboxylic acid		Quaternary amine		Sulfonic acid
Protein binding capacity per mL of polymer	18 mg/mL BSA	18 mg/mL BSA	23 mg/mL Lysozyme	23 mg/mL Lysozyme	18 mg/mL BSA	18 mg/mL BSA	30 mg/mL Lysozyme
Protein binding capacity per column	13 mg BSA	0.7 mg BSA	16 mg Lysozyme	0.9 mg Lysozyme	13 mg BSA	0.7 mg BSA	21 mg Lysozyme
Bed Height	44 mm	50 mm	42 mm	50 mm	45 mm	50 mm	42 mm
Bed volume	0.73 mL	0.039 mL	0.70 mL	0.039 mL	0.75 mL	0.039 mL	0.70 mL
pH Range	2 - 12						
Recommended Flow rate	0.5 – 1.5 mL/min	0.05 – 0.25 mL/min	0.5 – 1.5 mL/min	0.05 – 0.20 mL/min	0.5 – 1.5 mL/min	0.05 – 0.25 mL/min	0.5 – 1.5 mL/min
Maximum Flow rate	2 mL/min	0.30 mL/min	2 mL/min	0.25 mL/min	2 mL/min	0.3 mL/min	2 mL/min
Operating pressure	< 500 psi 3.4 MPa	< 1500 psi 10.4 MPa	< 500 psi 3.4 MPa	< 1000 psi 6.9 Mpa	< 500 psi 3.4 MPa	< 1500 psi 10.4 MPa	< 500 psi 3.4 MPa
Maximum Pressure	1000 psi 6.9 MPa	2000 psi 13.8 MPa	1000 psi 6.9 Mpa	2000 psi 13.8 Mpa	1000 psi 6.9 MPa	2000 psi 13.8 MPa	1000 psi 6.9 Mpa
Temperature	60 °C				70 °C		60 °C
Solvent Compatibility	Most common organic solvents						

Microchip with Particular Column Bed





35 µm o.d. electrospray tip

Microchannel slurry packed with spherical particles

Wirth, Anal. Chem. 79 (2007) 801

Three-layer, laminated polyimide microchip, channel 50 µm depth, 75 µm width, 50-150 mm length

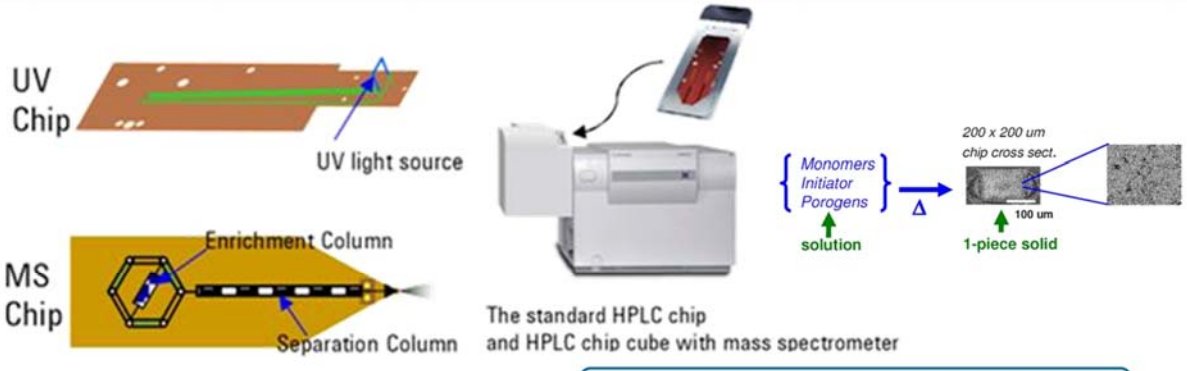
Yin, J. Sep. Sci. 30 (2007) 1427

Monolithic Separation Media in Agilent's HPLC-Chips

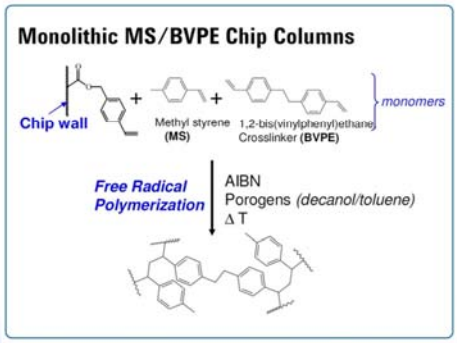
P-2701-W
HPLC'08

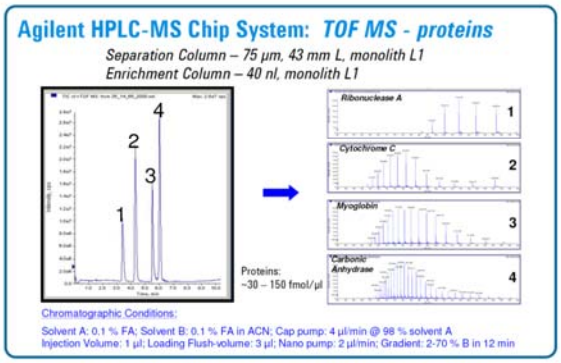
Karla M. Robotti[1], Hongfeng Yin[1], Reid Brennen[1], Kevin Killeen[1], Lukas Trojer[2], Günther Bonn[3]
[1] Agilent Technologies, Inc, Santa Clara, CA; [2] Agilent Technologies GmbH, Waldbronn, Germany;
[3] Institute of Analytical Chemistry and Radiochemistry, Leopold-Franzens University, Innsbruck, Austria

Agilent Technologies

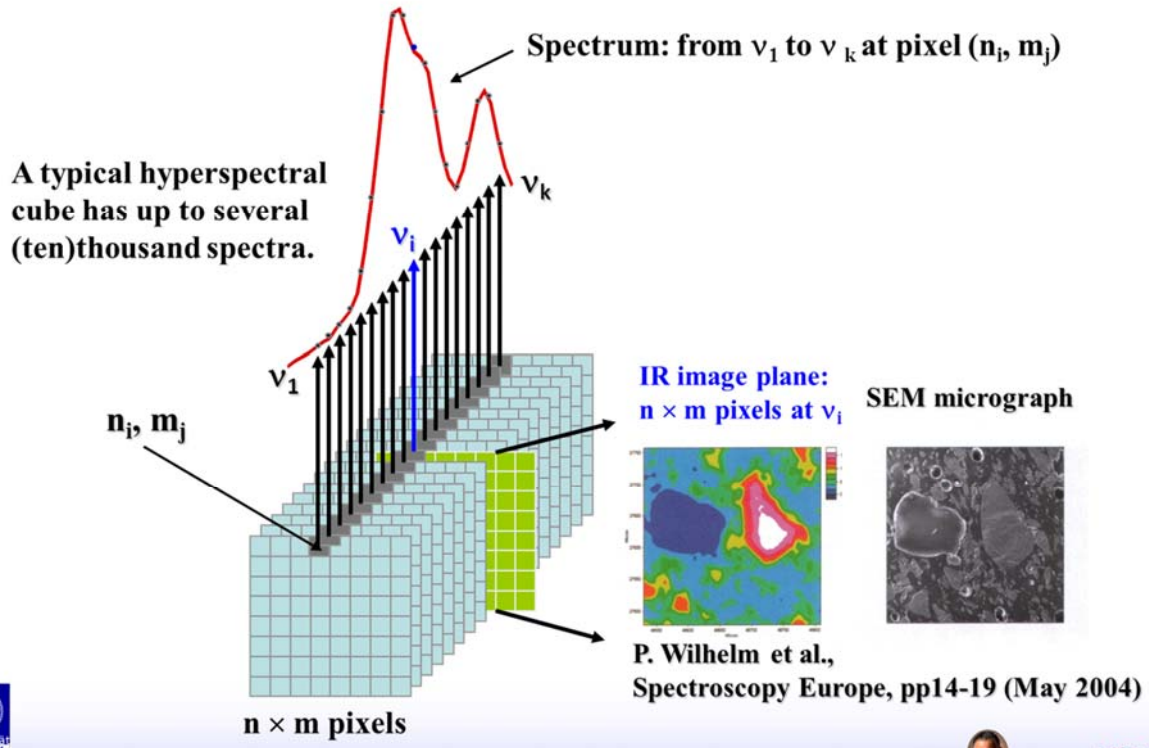


The standard HPLC chip and HPLC chip cube with mass spectrometer



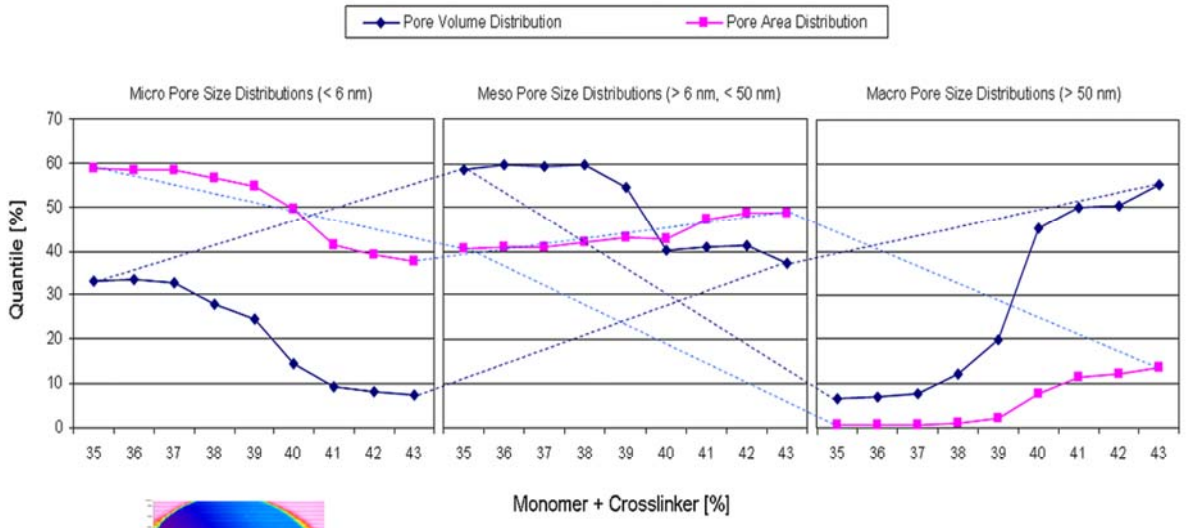


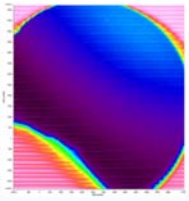
Characterization of Monolithic MS/BVPE capillaries by hyperspectral infrared imaging



P. Wilhelm et al.,
Spectroscopy Europe, pp14-19 (May 2004)

Characterization of Monolithic MS/BVPE capillaries by hyperspectral infrared imaging





Resulting pore volume and pore area distributions by gradually increasing the MS/BVPE content. Note that not the entire macropore range can be assessed with nitrogen adsorption (only up to d ~ 200 nm).

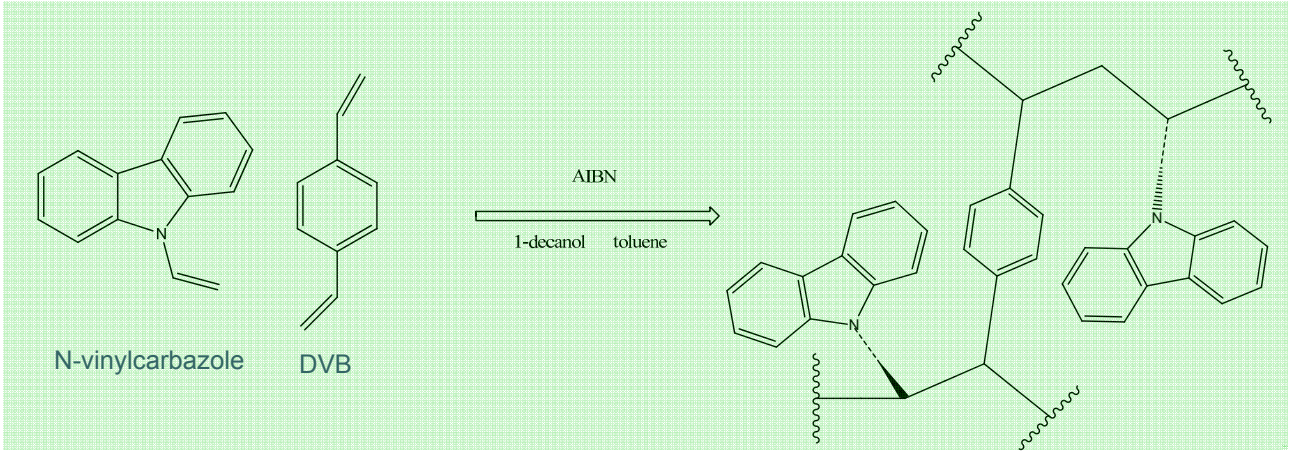
Institute of Analytical Chemistry and Radiochemistry, University of Innsbruck

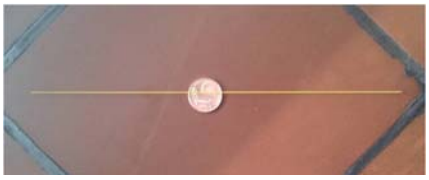
New Poly (N-vinylcarbazole -co- divinylbenzene) (NVC/DVB)

optimized for the separation of

Macromolecules

Poly (N-vinylcarbazole -co- divinylbenzene)





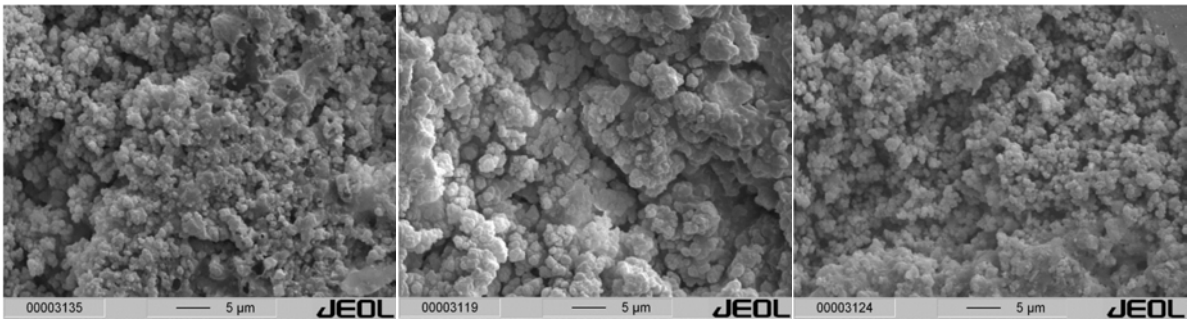
Capillary columns
50-200µm I.D.

Köck, Rainer, et al. "Monolithic Poly (N-vinylcarbazole-co-1, 4-divinylbenzene) Capillary Columns for the Separation of Biomolecules." Analyst (2013) in press

Institute of Analytical Chemistry and Radiochemistry, University of Innsbruck

Poly (N-vinylcarbazole -co- divinylbenzene)

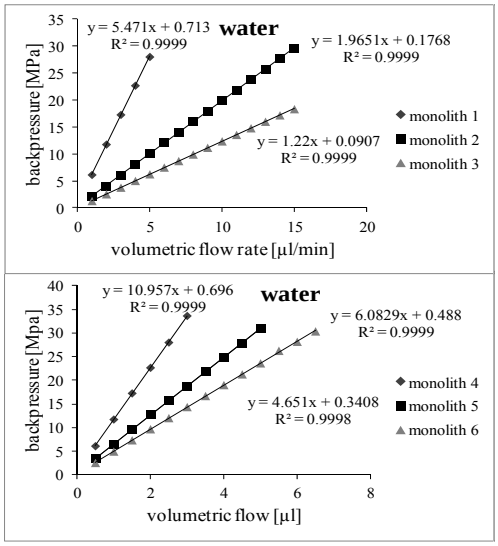
Scanning electron microscope



Mixture 1	Mixture 2	Mixture 3
37% monomers (3:2) 50% macroporogen	37% monomers (1:1) 51% macroporogen	37% monomers (3:2) 55% macroporogen

Poly (N-vinylcarbazole -co- divinylbenzene)

Hydrodynamic properties



Composition of monolithic columns 1-6

Monolith number	Column dimension (mm)	Monomer (%v/v)	NVC (%v/v)	DVB (%v/v)	Macroporogen (%v/v)	Microporogen (%v/v)
1	70 x 0.2	37.0	22.2	14.8	50.0	13.0
2	70 x 0.2	37.0	22.2	14.8	51.0	12.0
3	70 x 0.2	37.0	22.2	14.8	52.0	11.0
4	70 x 0.1	37.0	22.2	14.8	50.0	13.0
5	70 x 0.1	37.0	22.2	14.8	51.0	12.0
6	70 x 0.1	37.0	22.2	14.8	52.0	11.0

1% w/v of AIBN was used as free radical initiator

Permeability for water: **1.27×10^{-14} m²** for 200µm i.d. and **7.66×10^{-15} m²** for 100µm i.d. capillary supports.

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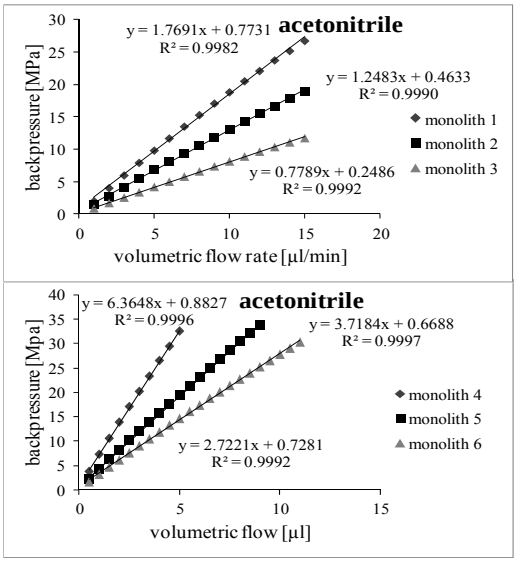
Poly (N-vinylcarbazole -co- divinylbenzene)

Hydrodynamic properties



Composition of monolithic columns 1-6

Monolith number	Column dimension (mm)	Monomer (%v/v)	NVC (%v/v)	DVB (%v/v)	Macroporogen (%v/v)	Microporogen (%v/v)
1	70 x 0.2	37.0	22.2	14.8	50.0	13.0
2	70 x 0.2	37.0	22.2	14.8	51.0	12.0
3	70 x 0.2	37.0	22.2	14.8	52.0	11.0
4	70 x 0.1	37.0	22.2	14.8	50.0	13.0
5	70 x 0.1	37.0	22.2	14.8	51.0	12.0
6	70 x 0.1	37.0	22.2	14.8	52.0	11.0

1% w/v of AIBN was used as free radical initiator

Permeability for water: **1.27x10^{-14} m²** for 200µm i.d. and **7.66x10^{-15} m²** for 100µm i.d. capillary supports. Followed by 21%, 26% and 41% reduced permeability for acetonitrile, methanol and tetrahydrofuran, respectively.

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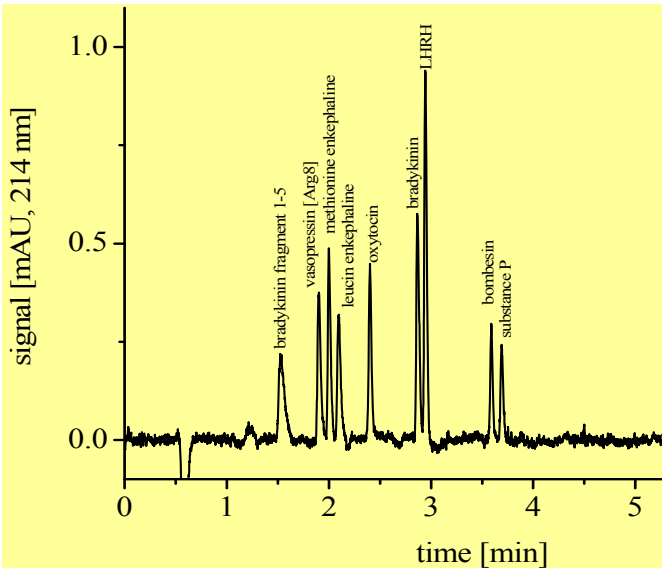
Highly efficienct separations of peptides

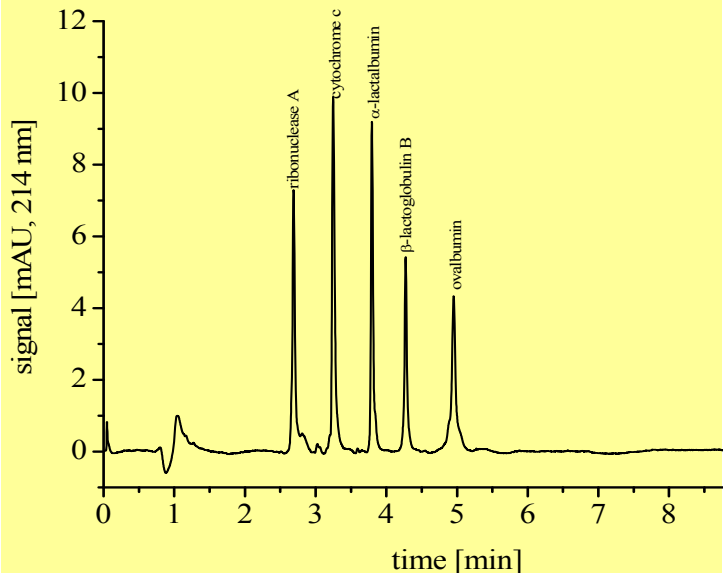


Chromatographic parameters

peptides	t_R [min]	$b_{0.5}$ [s]	R
bradykinin fragment 1-5	1.537	3.60	4.60
methionine enkephaline	1.903	2.03	1.79
leucin enkephaline	1.999	1.79	1.77
oxytocin	2.097	2.11	5.74
bradykinin	2.402	1.65	9.17
LHRH	2.864	1.92	1.59
bombesin	2.944	1.64	14.61
substance P	3.590	1.49	2.21
	3.691	1.74	

Gradient separation of peptides: 70 mm x 0.1 mm capillary column; Separation conditions: 1-30% B in 5 min (0.1%TFA in water and ACN) , 70° C, UV 214 nm, inj.:100 nl, 0.2 ng each peptide, flow rate: 4 µl/min

Highly efficienct separations of proteins

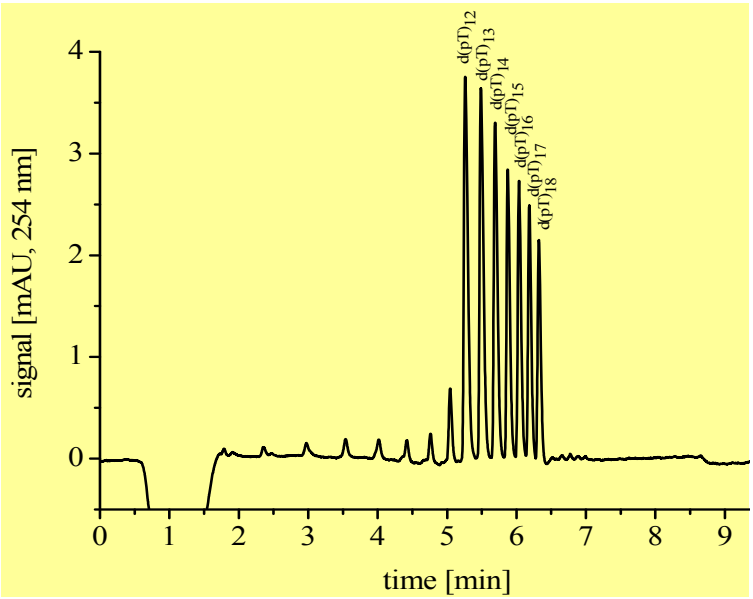


Chromatographic parameters

proteins	t_R [min]	$b_{0.5}$ [s]	R
ribonuclease A	2.688	2.11	8.86
cytochrome c	3.247	2.35	9.18
α-Lactalbumin	3.795	1.88	8.75
β-Lactoglobulin B	4.273	1.99	9.46
ovalbumin	4.953	3.10	

Gradient separation of proteins: 70 mm x 0.1 mm capillary column; Separation conditions: 15-65% B in 5 min (0.1%TFA in water and ACN) , 3 µl/min, 60° C, UV 214 nm, inj.:100 nl, 1ng each protein.

Highly efficienct separations of oligonucleotides



Ion-pair Reversed Phase

Chromatographic parameters

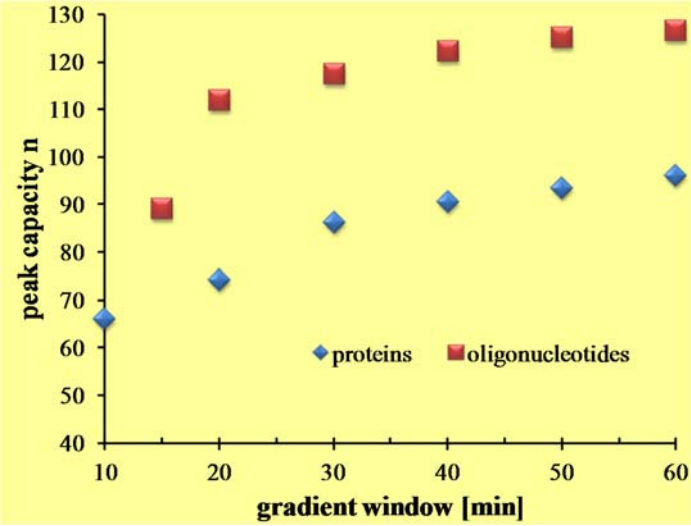
oligonucleotides	t_R [min]	$b_{0.5}$ [s]	R
$d(pT)_{12}$	5.262	3.87	2.10
$d(pT)_{13}$	5.487	3.73	2.03
$d(pT)_{14}$	5.692	3.42	1.96
$d(pT)_{15}$	5.875	3.19	1.82
$d(pT)_{16}$	6.037	3.09	1.73
$d(pT)_{17}$	6.185	2.98	1.63
$d(pT)_{18}$	6.322	2.94	

Gradient separation of oligonucleotides: 70 mm x 0.2 mm capillary column. Separation conditions: 15-40% ACN in 7 min (0.1 M TEAA), 4 µl/min, 50° C, UV 254 nm, inj.: 1 µl, 5 ng total.

Poly (N-vinylcarbazole -co- divinylbenzene)

Peak capacity in gradient elution

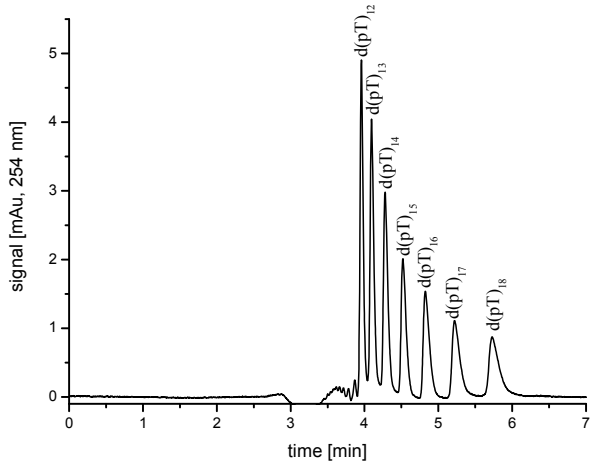
$$PC = 1 + \frac{t_G}{W_{av}}$$



Peak capacity plots of gradient separations of proteins and oligonucleotides. Separation conditions proteins (RP): 70 mm x 0.1 mm NVC/DVB monolith; 15-50% B in 10-60 min, 4 µl/min, 70° C, UV 214 nm, inj.:300 nl, 1ng each protein. Separation conditions oligonucleotides (IP-RP): 70 mm x 0.1 mm NVC/DVB monolith; 20-70% B in 15-60 min (B=0.1 M TEAA pH 7 in 20 % acetonitrile), 50° C, UV 254 nm, inj.: 300 nl, 5 ng total $d(pT)_{12-16}$.

Poly (N-vinylcarbazole -co- divinylbenzene)

Isocratic separation of oligonucleotides pushed to the limit



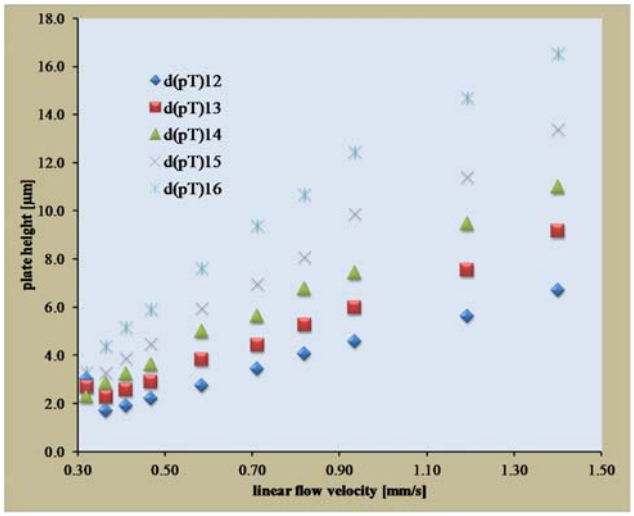
Van Deemter plots of $d(pT)_{12}$ to $d(pT)_{16}$ employing isocratic ion-pair reversed-phase conditions (IP-RP): 70 mm x 0.1 mm capillary column. Separation conditions: 0.8 µl/min; isocratic elution 0.1 M TEAA pH 7 in 10 % acetonitrile, 50° C, UV 254 nm (3nl cell), inj.: 300 nl, 3 ng total.

- applying maximum elution strenght still assuring resolution >1.5 for all peaks
- no retention on commercially available monolithic counterparts

oligonucleotides	$b_{0.5}$ [s]	R
$b_{0.5}$ $d(pT)_{12}$	2.76	1.60
$b_{0.5}$ $d(pT)_{13}$	3.30	1.83
$b_{0.5}$ $d(pT)_{14}$	3.84	2.07
$b_{0.5}$ $d(pT)_{15}$	4.38	2.21
$b_{0.5}$ $d(pT)_{16}$	5.40	2.34
$b_{0.5}$ $d(pT)_{17}$	6.60	2.43
$b_{0.5}$ $d(pT)_{18}$	8.28	

Poly (N-vinylcarbazole -co- divinylbenzene)

Van Deemter plot – oligonucleotides



u [mm/s]	t_R d(pT)$_{12}$ [s]	$b_{0.5}$ d(pT)$_{12}$ [s]	N (d(pT)$_{12}$) per column	N (d(pT)$_{12}$) per meter	plate height [µm]
1.40	70.32	1.62	10438	149121	6.7
1.19	82.38	1.74	12418	177401	5.6
0.93	100.92	1.92	15306	218657	4.6
0.82	113.58	2.04	17173	245333	4.1
0.71	130.80	2.16	20315	290215	3.4
0.58	154.62	2.28	25478	363977	2.7
0.47	190.68	2.52	31719	453128	2.2
0.41	210.60	2.58	36914	527338	1.9
0.36	237.48	2.76	41015	585933	1.7
0.32	305.52	4.73	23114	330194	3.0

New Poly (N-vinylcarbazole -co- divinylbenzene) (NVC/DVB)

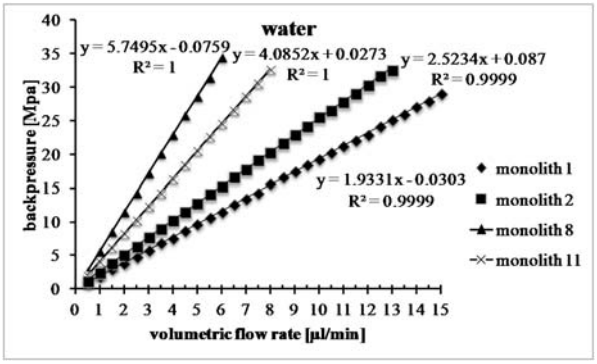
optimized for the separation of

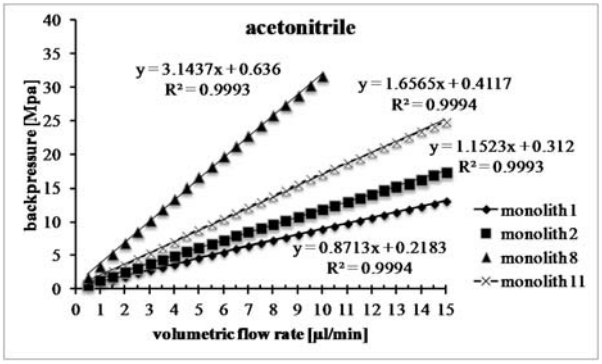
Small Molecules

Institute of Analytical Chemistry and Radiochemistry, University of Innsbruck

Poly (N-vinylcarbazole -co- divinylbenzene)

Hydrodynamic properties



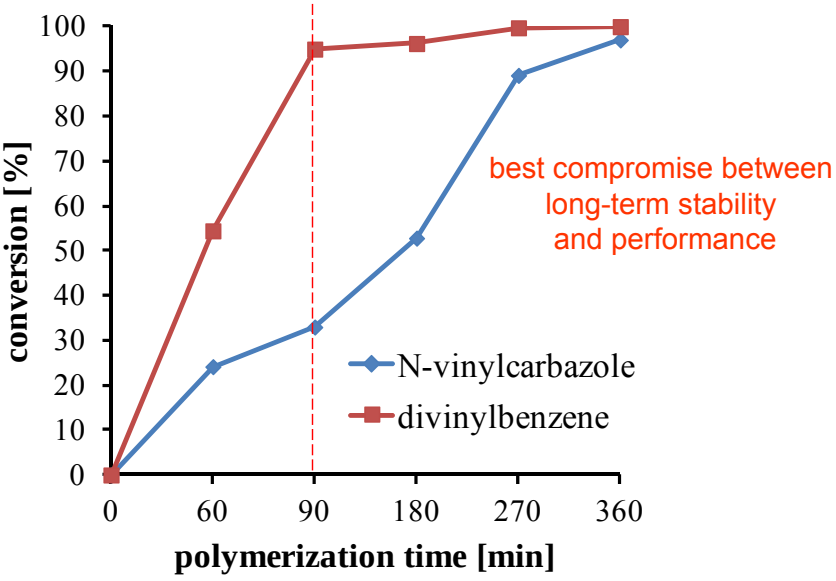


Monolith number	Column dimension (mm)	AIBN (%w/v)	Monomer (%v/v)	NVC (%v/v)	DVB (%v/v)	Macroporogen (%v/v)	Microporogen (%v/v)	Polymerization time (min)
1	80 x 0.20	2.0	38.0	19.0	19.0	51.0	11.0	90.0
2	80 x 0.20	2.0	38.0	19.0	19.0	50.0	12.0	90.0
3	80 x 0.20	2.0	38.0	19.0	19.0	52.0	50.0	90.0
4	80 x 0.20	2.0	39.0	19.5	19.5	50.0	11.0	90.0
5	80 x 0.20	2.0	39.0	19.5	19.5	51.0	10.0	90.0
6	80 x 0.20	2.0	38.0	19.0	19.0	52.0	11.0	180.0
7	80 x 0.20	2.0	38.0	19.0	19.0	51.0	11.0	270.0
8	80 x 0.20	2.0	38.0	19.0	19.0	51.0	11.0	360.0
9	80 x 0.20	1.5	38.0	19.0	19.0	51.0	11.0	90.0
10	80 x 0.20	2.5	38.0	19.0	19.0	51.0	11.0	90.0
11	80 x 0.20	3.0	38.0	19.0	19.0	51.0	11.0	90.0
	150 x 0.05	2.0	38.0	19.0	19.0	51.0	11.0	90.0

The NVC/DVB monolithic stationary phase showed the highest relative permeability for water, 1.11E-14 m^2, applying monolith 1. For acetonitrile, methanol and tetrahydrofurane, the permeability was reduced by 10%, 26% and 41%, respectively.

Poly (N-vinylcarbazole -co- divinylbenzene)

Monomer conversion

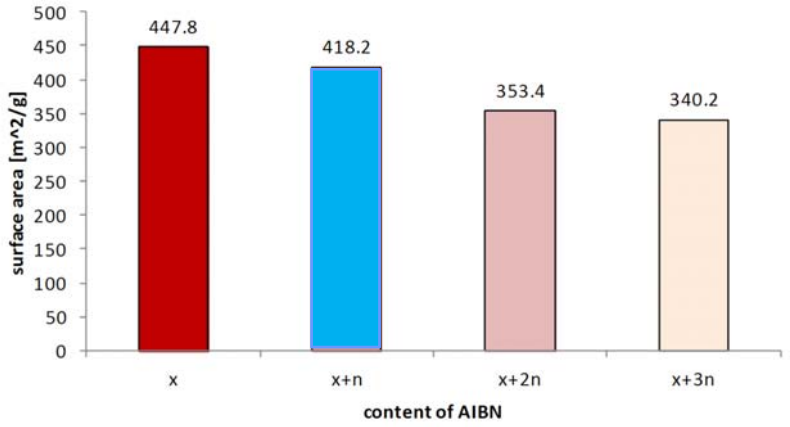


Monomer conversion analyzed by GC-MS

Poly (N-vinylcarbazole -co- divinylbenzene)

BET – surface area

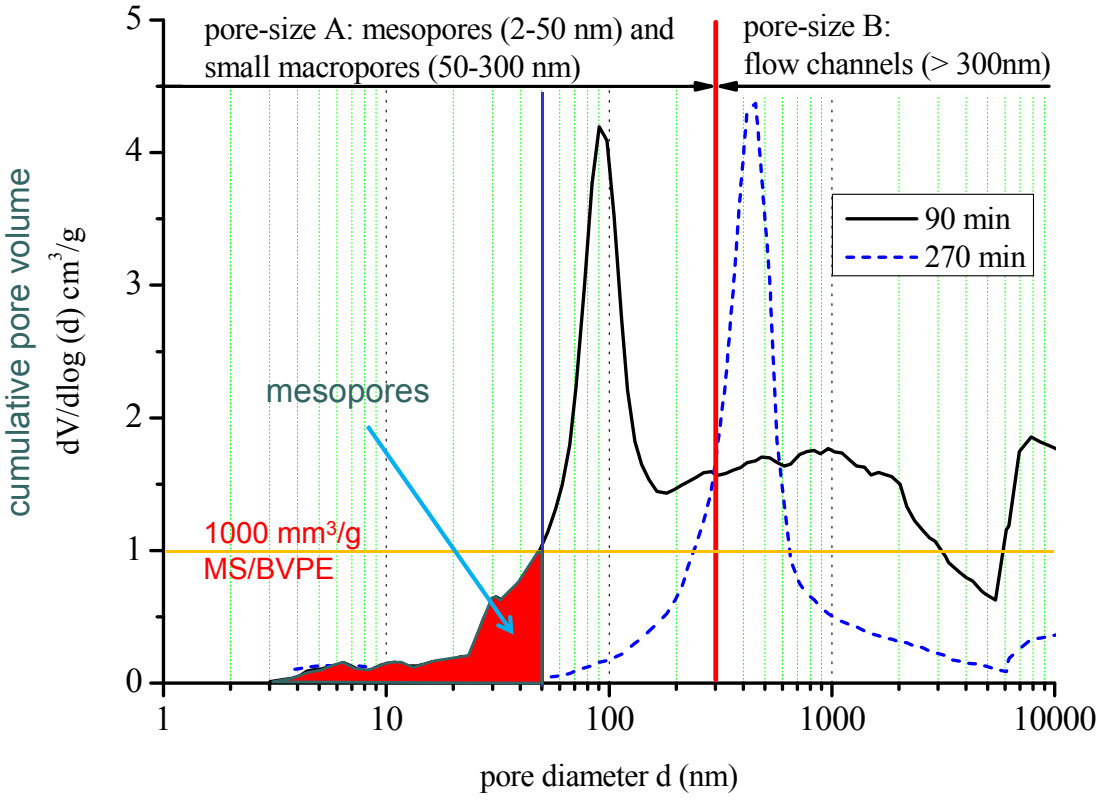
- Poly (NVC/DVB) combines high surface areas with ease of production



- MS/BVPE ≈ 75 m^2/g (L. Trojer[1] (2009))
- PS/DVB ≈ 100 m^2/g (I. Nischang[2] (2010))
- PS/VBCl/DVB with hypercrosslinking ≈ 631 m^2/g (F. Svec[3] group(2010))

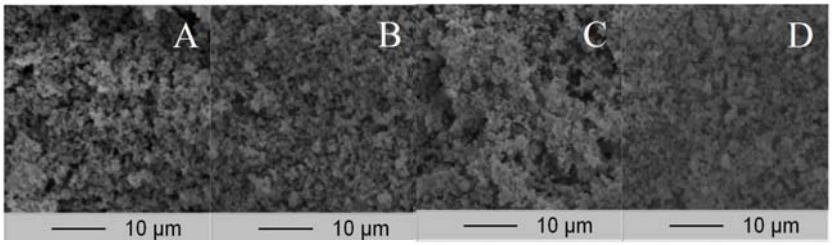
1. L. Trojer, C. P. Bisjak, W. Wieder, G. K. Bonn, *J. Chromatogr. A* 1216 (2009) 6303.
2. I. Nischang, I. Teasdale, O. Bruggemann, *J. Chromatogr. A* 1217 (2010) 7514.
3. J. Urban, F. Svec, J.M.J. Fréchet, *J.Chromatogr. A* 1217 (2010) 8212.

Pore-size distribution profile of Poly(NVC-DVB)



Poly (N-vinylcarbazole -co- divinylbenzene)

Scanning electron microscope



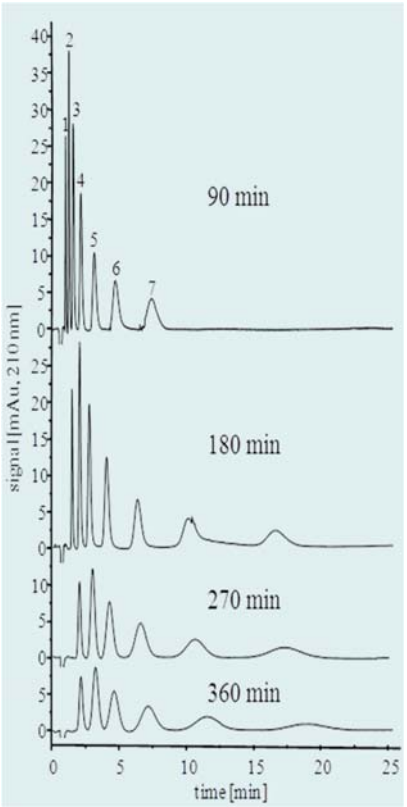
Characterization of surface morphology by scanning electron microscope (SEM) images. Four different polymer mixtures:
(A) monolith 1 (Table, entry 1),
(B) monolith 4 (Table, entry 4)
(C) monolith 8 (Table 1, entry 8)
(D) monolith 11 (Table, entry 11).

Monolith number	Column dimension (mm)	AIBN (%w/v)	Monomer (%v/v)	NVC (%v/v)	DVB (%v/v)	Macroporogen (%v/v)	Microporogen (%v/v)	Polymerization time (min)
1	80 x 0.20	2.0	38.0	19.0	19.0	51.0	11.0	90.0
2	80 x 0.20	2.0	38.0	19.0	19.0	50.0	12.0	90.0
3	80 x 0.20	2.0	38.0	19.0	19.0	52.0	50.0	90.0
4	80 x 0.20	2.0	39.0	19.5	19.5	50.0	11.0	90.0
5	80 x 0.20	2.0	39.0	19.5	19.5	51.0	10.0	90.0
6	80 x 0.20	2.0	38.0	19.0	19.0	52.0	11.0	180.0
7	80 x 0.20	2.0	38.0	19.0	19.0	51.0	11.0	270.0
8	80 x 0.20	2.0	38.0	19.0	19.0	51.0	11.0	360.0
9	80 x 0.20	1.5	38.0	19.0	19.0	51.0	11.0	90.0
10	80 x 0.20	2.5	38.0	19.0	19.0	51.0	11.0	90.0
11	80 x 0.20	3.0	38.0	19.0	19.0	51.0	11.0	90.0
12	150 x 0.05	2.0	38.0	19.0	19.0	51.0	11.0	90.0

Poly (N-vinylcarbazole -co- divinylbenzene)

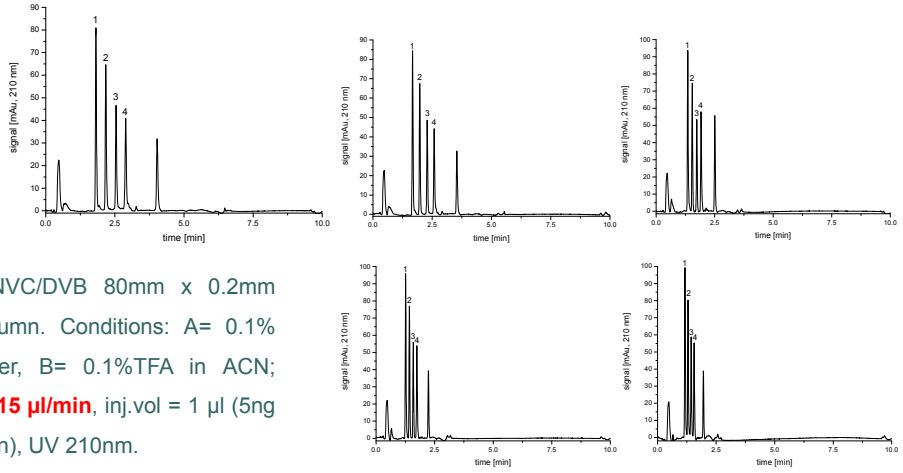
Different polymerization times – impact on efficiency?

Influence of the polymerization time on the isocratic separation of alkylbenzenes.



Poly (N-vinylcarbazole -co- divinylbenzene)

Gradient separations of parabens

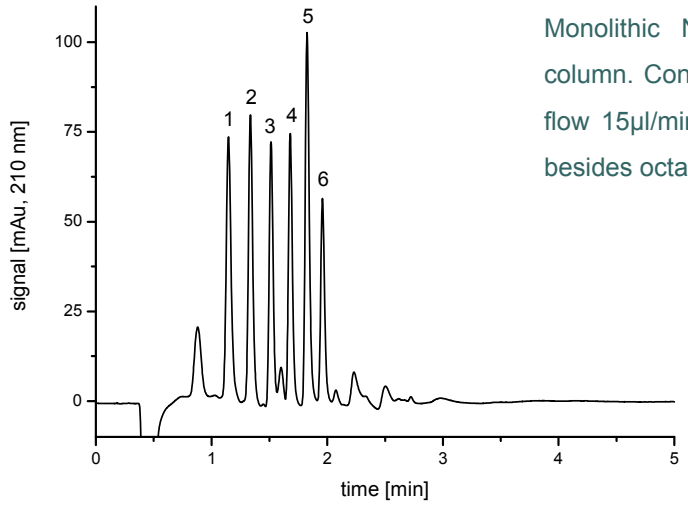


Monolithic NVC/DVB 80mm x 0.2mm capillary column. Conditions: A= 0.1% TFA in water, B= 0.1%TFA in ACN; 70° C, flow **15 µl/min**, inj.vol = 1 µl (5ng each paraben), UV 210nm.

gradient	t_R [min]	$b_{0.5}$ [s]	R	As	t_R [min]	$b_{0.5}$ [s]	R	As	t_R [min]	$b_{0.5}$ [s]	R	As	t_R [min]	$b_{0.5}$ [s]	As
	methylparaben				ethylparaben				propylparaben				butylparaben		
1-99 % B in 6 min	1.815	2.54	4.88	1.23	2.172	2.63	4.95	1.22	2.543	2.69	4.33	1.20	2.892	3.00	1.45
1-99 % B in 5 min	1.673	2.42	4.34	1.20	1.975	2.50	4.41	1.21	2.288	2.53	3.96	1.19	2.583	2.74	1.47
1-99 % B in 3 min	1.353	2.21	3.01	1.32	1.543	2.26	3.05	1.14	1.738	2.27	2.78	1.15	1.920	2.36	1.43
1-99 % B in 2.5 min	1.263	2.18	2.56	1.30	1.423	2.24	2.58	1.23	1.588	2.28	2.38	1.20	1.743	2.34	1.19
1-99 % B in 2 min	1.168	2.14	2.13	1.31	1.298	2.19	2.17	1.24	1.433	2.21	1.97	1.10	1.558	2.28	1.14

Poly (N-vinylcarbazole -co- divinylbenzene)

Separation of acetophenones

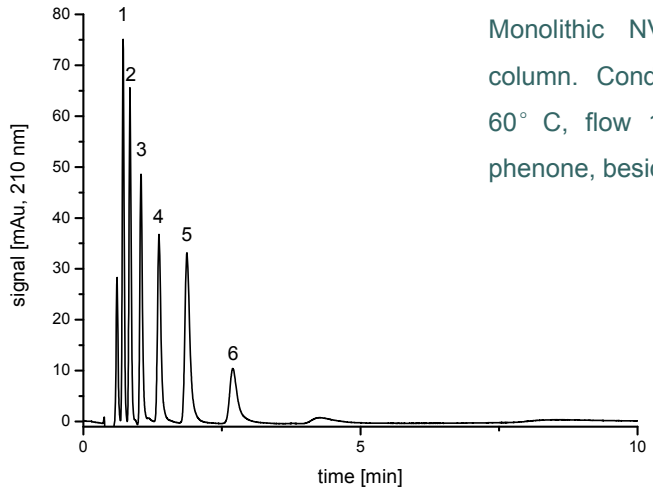


Monolithic NVC/DVB 80mm x 0.2mm capillary column. Conditions: 30-99% ACN in 2 min, 60° C, flow 15µl/min, inj.vol = 1 µl (2.5ng each phenone, besides octanophenone 5ng) , UV 210nm.

acetophenones	t_R [min]	$b_{0.5}$ [s]	R	As
propiophenone	1.147	2.65	2.67	1.20
butyrophenone	1.337	2.38	2.77	1.19
valerophenone	1.515	2.17	2.69	1.07
hexanophenone	1.680	2.17	2.33	1.25
heptanophenone	1.825	2.24	2.17	1.28
octanophenone	1.958	2.12	1.25	

Poly (N-vinylcarbazole -co- divinylbenzene)

Isocratic separation of acetophenones

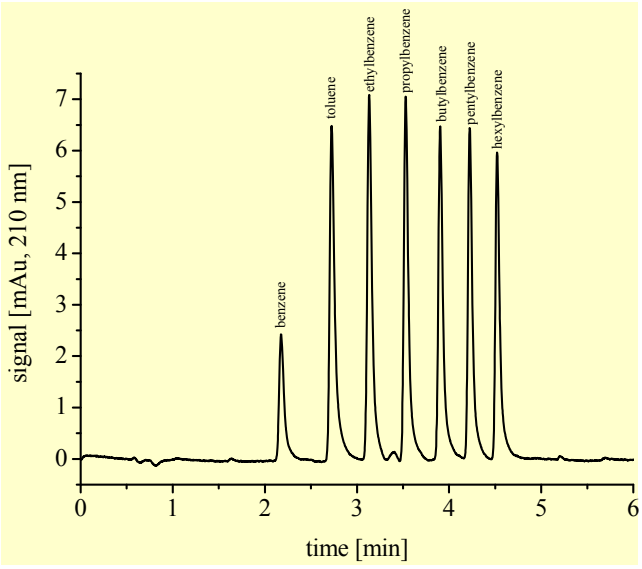


Monolithic NVC/DVB 80mm x 0.2mm capillary column. Conditions: isocratic elution 55 % ACN, 60° C, flow 15µl/min, inj.vol = 1 µl (2.5ng each phenone, besides octanophenone 5ng) , UV 210nm.

55% B acetophenones	t_R [min]	$b_{0.5}$ [s]	R	As
propiophenone	0.718	2.23	1.85	1.23
butyrophenone	0.842	2.48	2.61	1.24
valerophenone	1.040	2.91	3.38	1.30
hexanophenone	1.365	3.90	3.37	1.40
heptanophenone	1.870	5.68	4.10	1.51
octanophenone	2.697	8.56	1.71	

Poly (N-vinylcarbazole -co- divinylbenzene)

Highly efficienct separations of alkylbenzenes



Chromatographic parameters

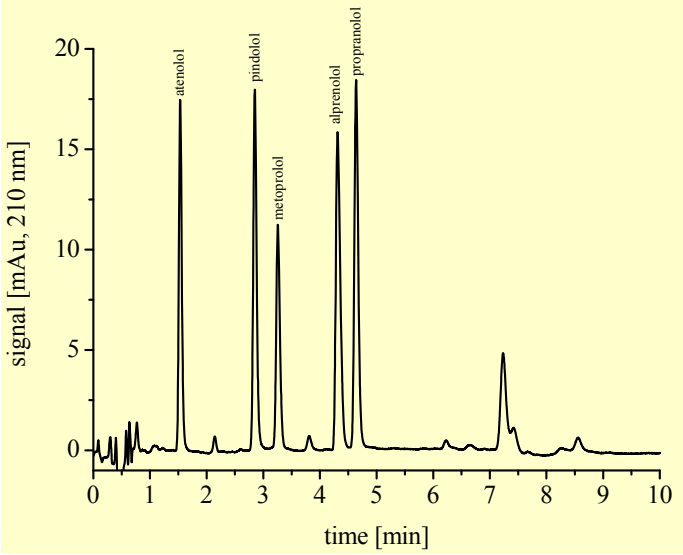
alkylbenzenes	t_R [min]	$b_{0.5}$ [s]	R
benzene	2.072	2.95	5.51
toluene	2.530	2.94	4.12
ethylbenzene	2.862	2.76	4.15
propylbenzene	3.178	2.64	4.05
butylbenzene	3.477	2.57	3.54
pentylbenzene	3.730	2.50	3.33
hexylbenzene	3.962	2.43	

Gradient separation of alkylbenzenes: 80 mm x 0.2 mm capillary column NVC/DVB. Separation conditions: 30-99% B in 5 min, 7 µl/min, 60° C, UV 210 nm (3nl cell), inj.: 500 nl, 3.5 ng each alkylbenzene.

Institute of Analytical Chemistry and Radiochemistry, University of Innsbruck

Poly (N-vinylcarbazole -co- divinylbenzene)

Highly efficienct separations of beta-blockers



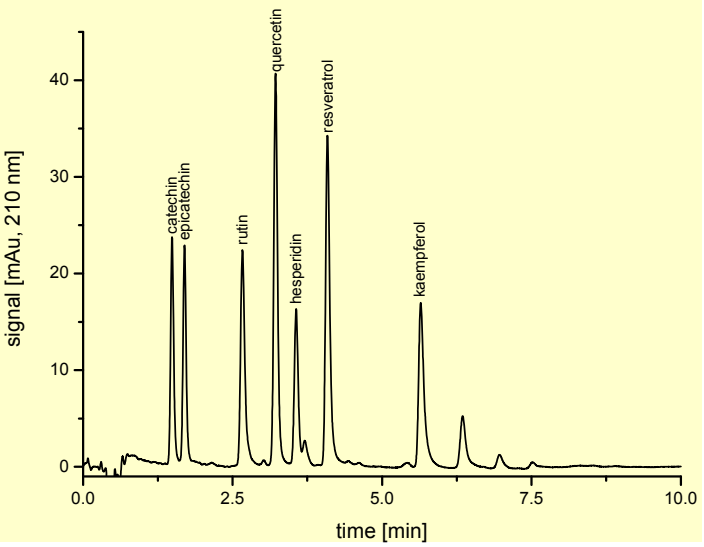
Chromatographic parameters

beta-blockers	t_R [min]	$b_{0.5}$ [s]	R
atenolol	1.533	3.33	13.09
pindolol	2.852	3.80	3.56
metoprolol	3.257	4.25	7.90
alprenolol	4.312	5.20	2.40
propranolol	4.638	4.42	

Gradient separation of beta-blockers: 80 mm x 0.2 mm capillary column NVC/DVB. Separation conditions: 1-40% ACN in 8 min, 15 µl/min, 40° C, UV 210 nm, inj.: 1 µl, 2 ng each beta-blocker.

Poly (N-vinylcarbazole -co- divinylbenzene)

Highly efficienct separations of flavanoids



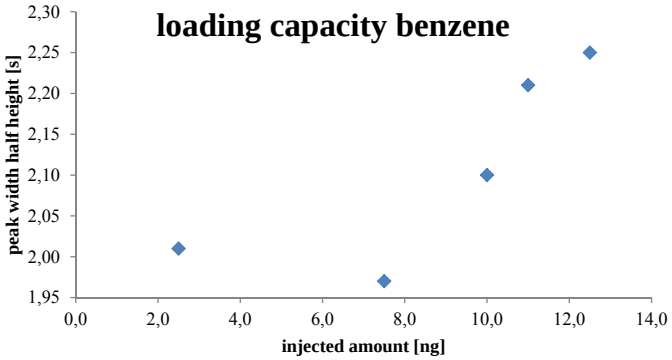
Chromatographic parameters

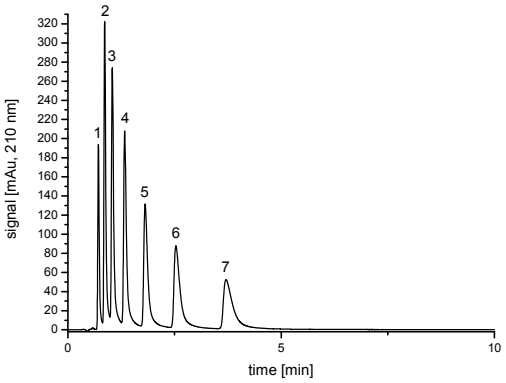
flavanoids	t_R [min]	$b_{0.5}$ [s]	R
catechin	1.487	3.32	2.24
epicatechin	1.697	3.33	8.81
rutin	2.665	4.45	4.63
quercetin	3.218	4.01	3.01
hesperidin	3.563	4.10	4.34
resveratrol	4.087	4.43	11.21
kaempferol	5.648	5.43	

Gradient separation of flavanoids/stilbenoid: 80 mm x 0.2 mm capillary column NVC/DVB. Separation conditions: 1-40% ACN in 8 min, 15 µl/min, 60° C, UV 210 nm, inj.: 1 µl, 3 ng each flavanoid/stilbenoid.

Poly (N-vinylcarbazole -co- divinylbenzene)

Loading Capacity

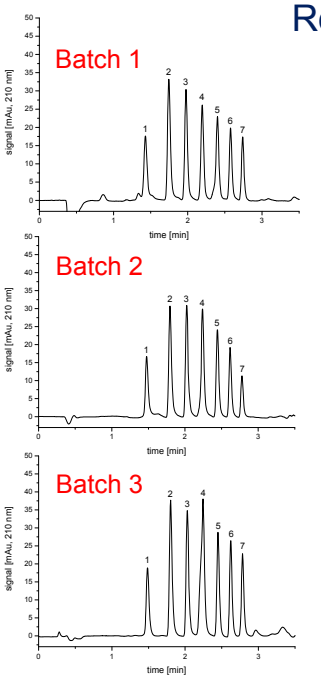




➢ the maximum loading capacity was reached when peak width at half height exceeds an arbitrarily set threshold of 10%. The capacity was found to be in the range of 77 ng alkylbenzenes!

Poly (N-vinylcarbazole -co- divinylbenzene)

Repeatability of the fabrication process

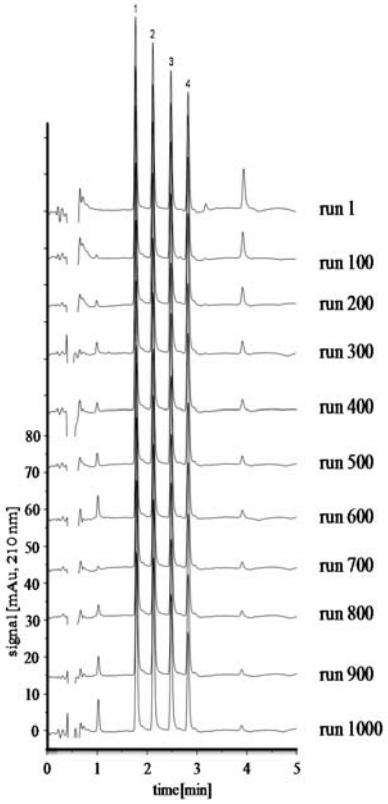


batch	t_R [min]	$b_{0.5}$ [s]	R	As	t_R [min]	$b_{0.5}$ [s]	R	As	t_R [min]	$b_{0.5}$ [s]	R	As	t_R [min]	$b_{0.5}$ [s]	R	As
	benzene				toluene				ethylbenzene				propylbenzene			
1	1.430	2.63	4.32	1.37	1.745	2.53	3.31	1.33	1.977	2.43	3.19	1.25	2.195	2.42	2.94	1.13
2	1.477	2.42	4.67	1.26	1.795	2.41	3.39	1.43	2.023	2.36	3.23	1.40	2.240	2.39	3.06	1.09
3	1.487	2.46	4.59	1.25	1.802	2.40	3.43	1.34	2.030	2.31	2.74	1.27	2.243	3.21	2.66	0.92
RSD %	2.1	4.5	4.1	5.1	1.7	3.0	1.8	4.0	1.4	2.5	8.9	6.2	1.2	17.4	7.1	10.7

	t_R [min]	$b_{0.5}$ [s]	R	As	t_R [min]	$b_{0.5}$ [s]	R	As	t_R [min]	$b_{0.5}$ [s]	As
	butylbenzene				pentylbenzene				hexylbenzene		
1	2.400	2.52	2.59	0.91	2.577	2.31	2.52	1.22	2.740	2.28	1.32
2	2.443	2.32	2.69	1.32	2.617	2.25	2.58	1.31	2.778	2.19	1.29
3	2.448	2.24	2.78	1.24	2.622	2.17	2.63	1.29	2.783	2.18	1.28
RSD %	1.1	6.1	3.5	18.8	0.9	3.1	2.1	3.7	0.8	2.5	1.6

Monolithic NVC/DVB 80mm x 0.2mm capillary column. Conditions: A= water, B= ACN; gradient 20-99%B in 3 min 50° C, flow 15µl/min, inj.vol = 1 µl (2.5ng each alkylbenzene) , UV 210nm.

Poly (N-vinylcarbazole -co- divinylbenzene)



Long-term stability-test

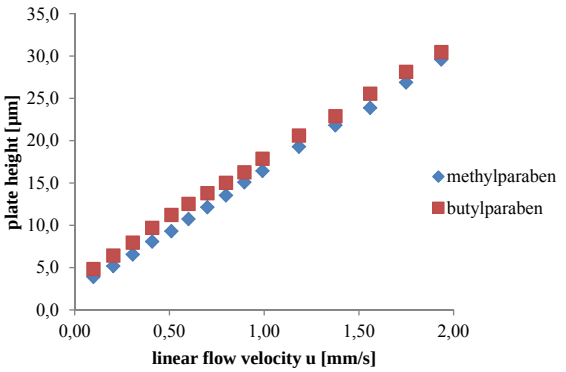
The chromatographic performance of the NVC/DVB stationary phase was evaluated on the long run by applying 1000 consecutive separations of four parabens with a gradient from 1-99% ACN in 5 min (0.1%TFA in water and ACN) , a flow rate of 15 µl/min, a column temperature of 70 ° C and 2 min equilibration time between runs. The maximum relative standard deviation was found to be 0.16, 0.85 and 0.97% RSD for retention times, peak width at half height and resolution, respectively. The excellent repeatability results during **1000 consecutive runs** attests high stability of the chromatographic supports without any noticeable performance reduction during this test procedure.

1 Methylparaben
2 Ethylparaben
3 Propylparaben
4 Butylparaben

Poly (N-vinylcarbazole -co- divinylbenzene)

Van Deemter plot – number of theoretical plates

Van Deemter plots of methylparaben and butylparaben employing isocratic reversed-phase conditions: 80 mm x 0.2 mm capillary column. Separation conditions: isocratic elution 65% ACN in water, room temperature, UV 210 nm, inj.: 1 µl, 4 ng total.



u [mm/s]	t_R methylparabene [s]	$b_{0.5}$ methylparabene [s]	R	As	N methylparabene per meter	plate height [µm]	t_R butylparabene [s]	$b_{0.5}$ butylparabene [s]	As	N butylparabene per meter	plate height [µm]
1.93	49.68	2.25	2.06	1.37	33793	29.6	58.32	2.68	1.30	32824	30.5
1.75	54.90	2.37	2.09	1.32	37194	26.9	64.08	2.83	1.34	35538	28.1
1.56	61.20	2.49	2.17	2.49	41873	23.9	71.28	3.00	3.00	39131	25.6
1.38	69.42	2.70	2.24	1.33	45821	21.8	80.58	3.21	1.31	43679	22.9
1.18	80.40	2.94	2.32	1.33	51837	19.3	93.12	3.52	1.35	48509	20.6
0.99	95.70	3.23	2.45	1.32	60848	16.4	110.52	3.89	1.33	55951	17.9
0.90	105.72	3.42	2.49	1.29	66235	15.1	121.50	4.08	1.33	61469	16.3
0.80	118.50	3.63	2.57	1.28	73867	13.5	136.02	4.39	1.36	66543	15.0
0.70	135.12	3.92	2.72	1.28	82355	12.1	155.22	4.80	1.34	72483	13.8
0.60	157.20	4.29	2.86	1.30	93071	10.7	180.48	5.32	1.36	79774	12.5
0.51	188.52	4.79	3.02	1.30	107367	9.3	216.18	6.03	1.35	89089	11.2
0.41	234.78	5.56	3.19	1.32	123594	8.1	268.68	6.97	1.39	102998	9.7
0.31	312.42	6.66	3.43	1.30	152530	6.6	355.98	8.36	1.36	125679	8.0
0.20	469.98	8.91	3.80	1.35	192854	5.2	535.02	11.29	1.46	155660	6.4
0.10	964.32	15.87	4.21	1.42	255926	3.9	1092.42	20.00	1.41	206797	4.8

Thermodynamics and Kinetics of LC

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JOURNAL OF CHROMATOGRAPHY A
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Review

Monolithic columns in high-performance liquid chromatography

Georges Guiochon [a,b,*]

[a] Department of Chemistry, University of Tennessee, Knoxville, TN 37996-1600, USA
[b] Division of Chemical Sciences, Oak Ridge National Laboratory, Oak Ridge, TN 37831-6120, USA

Available online 3 June 2007

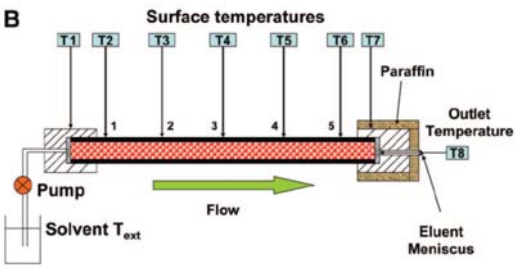
Experimental and theoretical studies of chromatographic processes.

Georges Guiochon

Thermodynamics and Kinetics of LC

Studying the different aspects of thermodynamics and kinetics of LC:

- measure single-component and competitive equilibrium isotherms
- study of the surface heterogeneity of adsorbents
- analysis of different parameters of the mass transfer kinetics and investigate the mechanisms involved, e.g., pore and surface diffusion
- study of elution band profiles of compounds from their equilibrium isotherms and mass transfer kinetics
- Measurement of the axial and radial temperature profiles of a chromatographic column; influence of thermal insulation on column efficiency



Conclusion - Monoliths

- Novel monolithic materials present reliable separation platforms for HPLC, since they offer high mechanical stability and minimised swelling properties.
- Careful development of the support porosity results capillary columns with enhanced hydrodynamic properties and separation efficiency
- Introduction of polymerisation time as novel polymerisation parameter offers a simple and effective tool to control mesoporosity of organic monoliths
- Adjusting meso- and macroporosity among each other widens up the field of applicability of organic monoliths
- Organic monoliths are excellent for SPE and bioreactor material

→ **Proteomic, Genomic as well as Metabolomic studies on one monolithic HPLC column!**

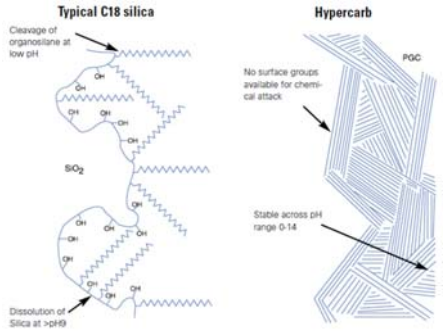
Overview

Characterisation by chemistry

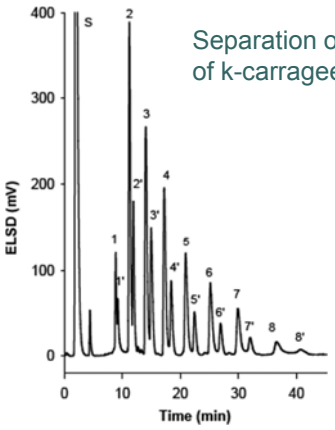
Silica-based (oxides)	Organic polymer-based	others e.g. carbon

Institute of Analytical Chemistry and Radiochemistry, University of Innsbruck

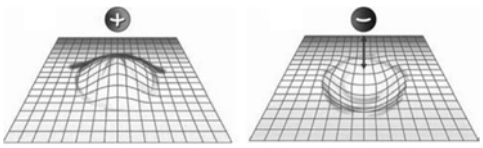
Porous Graphitic Carbon



Surface Comparison Between C18 Bonded Silica and Hypercarb Porous Graphitic Carbon



Separation of oligomers of k-carrageenan



Schematic representation of polar analyte retention in which (a) positive charge and (b) negative charges approach the graphite surface, resulting in a charge-induced dipole at the graphite surface

M.T. Gilbert, J.H. Knox and B. Kaur. **Porous glassy carbon, a new columns packing material for gas chromatography and high-performance liquid chromatography,** *Chromatographia* 16(1982), 138-146

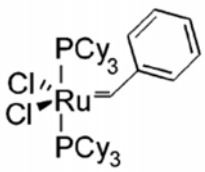
Pereira L, **Porous Graphitic Carbon as a Stationary Phase in HPLC: Theory and Applications** *Journal of Liquid Chromatography & Related Technologies* 31 (2008) 1687–1731.

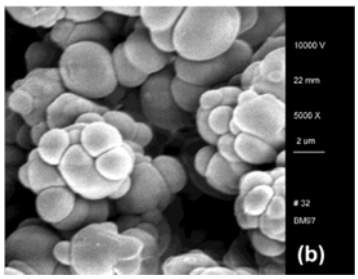
Special Polymerization Techniques

Ring opening metathesis polymerisation (ROMP) of norbon-2-ene and 1,4,4a,5,8,8a-hexahydro-1,4,5,8-exo,endo-dimethanonaphtalene initiated by a Grubbs type catalyst.

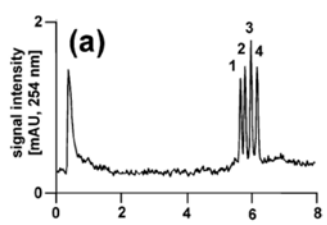
Monomers

Initiator

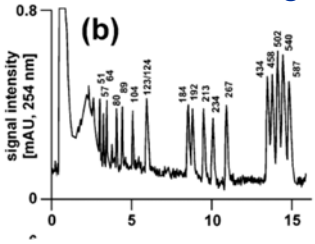




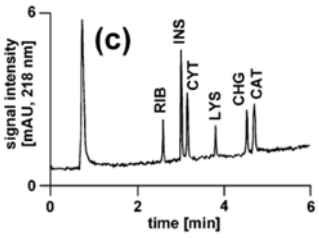
oligodeoxynucleotides



double-stranded DNA fragments



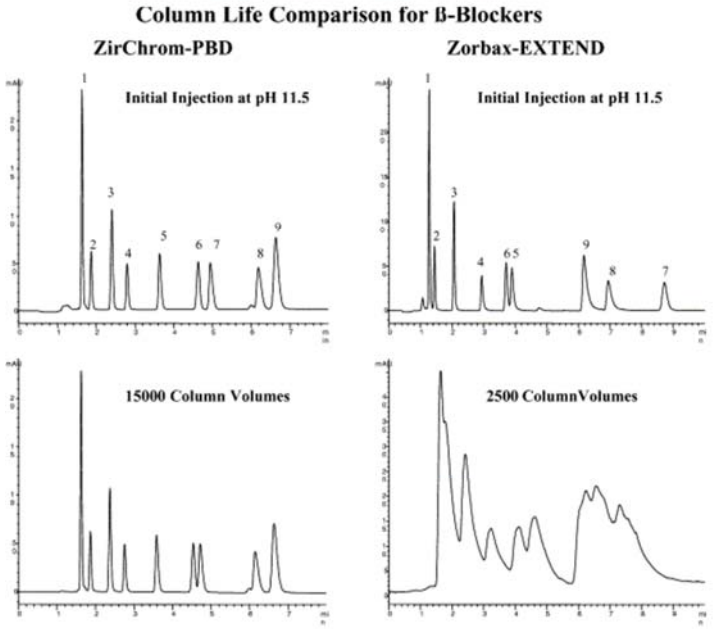
proteins



B. Mayr, G. Holzl, K. Eder, M.R. Buchmeiser, C.G. Huber. Anal. Chem. (2002), 74, 6080–6087.

Metal-Oxides

Titania, Zirconia, Alumina



J. Nawrocki, C. Dunlapb, A. McCormickc and P. W. Carrd, Journal of Chromatography A Vol. 1028, Issue 1 (2004), 1-30

Advantages

Extremely high thermal stability
High stability at all pHs

Reversed phases commercially available (with different to siliceous supports chemistry)
Well understood chemistry of zirconia's surface (surface chemistry of alumina and titania seems to be similar)
Similar (PBD-modified oxides vs. ODS silica) selectivity for non-electrolytes
Allow for a development of high temperature, organicless mobile phase, fast HPLC
Selectivity tunable by choice of buffer type

Very different selectivity compared to silica for basic and acidic compounds

Disadvantages

Available in limited selection of surface areas and pore sizes
Very different surface chemistry than that of silica especially Lewis acid interactions
Alumina and titania—relatively unexplored for HPLC

Lack of commercially available reversed phase for titania

Must have hard Lewis in eluent to separate carboxylics acids or else get no elution or extreme tailing
Few manufacturers

Effective use requires "unlearning" much knoweldge based on silica chemistry

Institute of Analytical Chemistry and Radiochemistry, University of Innsbruck