Clemens Banko

„Herstellung von stationären Phasen zur Reinigung von Feinchemikalien und pharmazeutischen Wirkstoffen mittels Elektrochromatographie“

Masterarbeit

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Abstract

This master thesis deals with the development and testing of C_8 – and aminofunctionalized silica monoliths, used in capillary electrochromatography (CEC). The goal is the qualitative and quantitative separation of different chemical mixtures. This requires good selectivity and short retention times.

The industrial approach is the separation and purification of pharmaceutical and basic chemical compounds, in a continuous annular chromatograph as developed in the *CAEC – Continuous Annular Electro-Chromatography Project in the 7th Framework Programme Theme 4 – NMP Nanosciences, Nanotechnologies, Materials and new Production Technologies* program.

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Further this thesis outlines the basic principles of chromatography and especially electrochromatography, the types of monoliths and the theory of their formation process. The manufactured functionalized silica monoliths were brought into 100 µm i.d. fused silica capillaries and tested with a variety of test mixtures, namely a polycyclic aromatic hydrocarbon (PAH) mixture, an alkylbenzene mixture and a phenol test mixture to determine the efficiency and selectivity of separation.

Additionally, the reproducibility of the capillaries regarding retention times was investigated. By altering the experimental conditions of the applied voltage and composition of the mobile phase the influence of these changes on the retention behaviour was studied. Also the used method in CEC was tested for its injection precision and reproducibility.

Further a BET analysis was carried out to determine the specific surface area of the manufactured monoliths. Completing the thesis a comparison between the manufactured columns and a commercial CEC capillary was carried out.

Kurzfassung

Diese Masterarbeit beschäftigt sich mit der Entwicklung und Herstellung von C_8 – bzw. aminofunktionalisierten Silika-Monolithen. Diese wurden in Kapillaren eingebracht und mit Hilfe der „Capillary Electro Chromatography“ (CEC) auf ihre qualitative und quantitative Trenneigenschaften getestet. Um dieses Ziel zu erreichen sind Selektivität und kurze Retentionszeiten vonnöten.

Hierzu wurden verschiedene Testmischungen verwendet. Die industrielle Verwendung dieser stationären Phasen wäre die Reinigung und Auftrennung verschiedener Pharmazeutika oder Basis – Chemikalien, in einem kontinuierlichen, annular aufgebauten Chromatographen wie er im Rahmen des *CAEC – Continuous Annular Electro-Chromatography Project in the 7th Framework Programme Theme 4 – NMP Nanosciences, Nanotechnologies, Materials and new Production Technologies* Programm entwickelt wurde.

Diese Arbeit wurde durch das 7th Research Framework Programme of the European Commission (Grant agreement number: NMP2-SL-2008-206707) unterstützt.

Weiters erläutert diese Masterarbeit die Prinzipien der Chromatographie, im speziellen die der Elektrochromatographie, die Monolith - Arten und die Theorie deren Herstellungsprozesses. Die hergestellten funktionalisierten Silika-Monolithe wurden in 100 µm Silika-Kapillaren eingebracht und mit verschiedenen Testmischungen (eine polyzyklische aromatische Kohlenwasserstoff (PAH) Probe, eine Alkylbenzol – Probe und eine Phenol Testmischung) auf die Effizienz und Selektivität der Trennung untersucht. Zusätzlich wurde die Reproduzierbarkeit der Kapillaren hinsichtlich Retentionszeiten eruiert. Neben der Validierung der CEC Methode betreffend der Präzision der Sample – Injektion sowie der Reproduzierbarkeit der Ergebnisse wurden die experimentellen Parameter (Zusammensetzung der mobilen Phase und angelegte Spannung) variiert und die Veränderungen bezüglich der Retentionszeiten dokumentiert. Außerdem wurde eine BET Analyse durchgeführt, um die spezifische Oberfläche des Monolithen zu bestimmen. Abschließend wird ein Vergleich der Effizienz der hergestellten stationären Phase mit der Effizienz von kommerziell erhältlichen CEC – Kapillaren dargestellt.

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Abbreviations

ACN	acetonitrile
API	active pharmaceutical ingredient
APTES	3-aminopropyltriethoxysilane
BET	Brunauer, Emmet and Teller calculations
c	concentration [$mol*L^{-1}$]
C_8TEOS	octyltriethoxysilane
CAC / CAEC	continuous annular electro - chromatography
CEC	capillary electro - chromatography
c_m	concentration of molecules in the mobile phase
c_s	concentration of molecules in the stationary phase
CTAB	hexadecyltrimethylammonium bromide
d	film thickness
d_C	diameter of the column
d_p	diameter of the particle
DVB	divinyl benzene
EC	electrochromatography
EOF	electroosmotic flow
EtOH	ethanol
E_λ	extinction at the specific wavelength λ
FID	flame ionization detector
G	Gibbs free energy [$kJ*mol^{-1}$]
GC	gas chromatography
H	plate height number
H_2O	(distilled) water
HCl	hydrochloric acid
HETP	height of an equivalent theoretical plate
HPLC	high pressure liquid chromatography
I	intensity of the transmitted light
I_0	intensity of the incident light
IEC	ion exchange chromatography
IR	infrared (light)
K	equilibrium constant
k’	capacity factor
k_c	rate constant for condensation
k_h	rate constant for hydrolysis
LC	liquid chromatography
L_c	column length
MeOH	methanol
N	plate number
n	mole
NaOH	sodium hydroxide

PAH	polycyclic aromatic hydrocarbons
PEG	polyethylene glycol
PSD	particle size distribution
RP	reversed phase
RS	chromatographic resolution parameter
SEC	size exclusion chromatography
SEM	scanning electron microscopy
t_0	dead time of the column
TEOS	tetraethoxysilane
TMOS	tetramethoxysilane
t_{plant}	dead time of the plant without the column
t_R	retention time
$t_{R\ net}$	net retention time
TRIS	tris(hydroxymethyl)aminomethane
t_{total}	total dead time
u_{int}	interstitial velocity
UV	ultraviolet (light)
V_{int}	interstitial volume of the fluid phase
VIS	visible light
V_m	volume of the mobile phase in a column
V_{pore}	volume of the pores
V_R	retention volume
V_s	volume of the stationary phase in a column
V_{solid}	volume of the solid material
V_{stat}	volume of the stationary phase
V_{total}	total volume of the column
$w_{1/2,\ i}$	peak width at half height
α	selectivity (= separation factor)
ε	void fraction
ε_{solid}	porosity of the solid phase
ε_{total}	total porosity
ε_λ	extinction coefficient at a specific wavelength λ
σ_t	standard deviation
Φ	phase ratio

1 Goals and Motivation

Chromatography is widely used in the pharmaceutical and chemical industry for purification and separation of active pharmaceutical ingredients (APIs). Although the method of chromatography is well investigated and understood, there are always new separation/purification problems, requiring a novel approach on both stationary and mobile phases as well as for the equipment used.

An innovative and promising field of chromatography is the electro-chromatography. Based on the principles of electrophoresis, electro-chromatography finds it´s way into pharmaceutical industry, aiming for a faster, cheaper and more efficient continuous separation and purification of basic pharmaceuticals and active pharmaceutical ingredients. Promoting this, the European Commission funded the *CAEC–Continuous Annular Electro-Chromatography Project in the 7th Framework Programme Theme 4–NMP Nanosciences, Nanotechnologies, Materials and new Production Technologies*. Eight reknown institutes and companies with the mutual interest in the purification of pharmaceuticals cooperate to alter the traditional batch procedure to continuous processes benefitting from the higher troughput without sacrificing efficiency. The partners are the Dortmund University of Technology, Graz University of Technology, University of Kaiserslautern, Institute of Microtechnology Mainz GmbH, Microinnova Engineering GmbH, Novartis Pharma AG, Technical University Eindhoven and Galileaus Oy.

The ultimate goal of the project is the development of a prototype with a (glass) annulus, containing a stationary phase dependent on the separation task. Feed is added at the top of the prototype, the annular angle and the applied voltage guarantee a high separation efficiency. Due to the complex nature of this task, the project was split into 8 work packages, making it easier to monitor the progress and maintaining the overview.

Work package 1 deals with the equipment design of the continuous annular electro chromatograph. The Kaiserslautern University of Technology leads this work package, the IMMs (Institut für Mikrotechnik Mainz GmbH) task is to design, manufacture, test and optimize the CAEC prototype with the parts applied by the other working packages.

In *Work package 2*, the Graz University of Technology, is responsible for the development, functionalization and characterization of the customized stationary phase. Additionally the stationary phase has to be inserted into capillaries.

Work package 3 is led by the University of Dortmund and is developing a predictive steady state model for progress design and optimization and a dynamic model for adaptive process control.

Work package 4 is also carried out at the University of Dortmund, which is in charge of developing on – line sensors to determine characteristic process and an adaptive process control strategy.

In *Work package 5* the Microinnova Engineering GmbH, is responsible for the integration of the prototype to establish a fully equipped CAEC unit.

In *work package 6*, all project partners are involved to validate the CAEC plant at industrial conditions.

Work package 7 deals with dissemination and exploitation tasks.

As for the management of the consortium there was established *Work package 8.*

The Institut für Mikrotechnik Mainz GmbH is involved in *work package 1, 4 and 6*, it also manufactures the CAEC prototype.

This master thesis covers the optimization and characterization of the stationary phases. For testing the stationary phases they are filled into CEC-capillaries. The detailed description for assembly and the results of the capillaries tested in Capillary Electro Chromatography (CEC) are mentioned in this thesis. Since a CEC device is not available at the IPPT the capillaries were tested at the workgroup of Hrn. Ao. Univ.-Prof. Dr. Martin Schmid of the Institut für Pharmazeutische Wissenschaften, Karl-Franzens University and in the workgroup of Hrn. Prof. Dipl.-Ing. Dr. techn Hans - Jörg Bart of the Lehrstuhl für Thermische Verfahrenstechnik, TU Kaiserslautern,

To synthesize the stationary phase a sol-gel process was used, based on the method described in the Diploma thesis of Marie Braunbruck.[1]

2 Introduction

2.1 Chromatography

Chromatography (derived from greek χρῶμα chroma "color" and γράφειν graphein "to write") is a set of laboratory techniques to separate mixtures.[2] The basic principle behind is the difference in a compounds partition (coefficient) between a mobile phase, in which the analyte is dissolved and a stationary phase, through which the mobile phase passes through. Due to different mechanisms (for detailed description see point 3.4) the analytes travel through the stationary phase (with specific physico-chemical properties which influences the separation and partition) is delayed, resulting in prolonged or shortened retention times of the compound analyzed.

Chromatography can both used preparative, meaning a separation of mixtures for further use is desired or analytical information about the sample is aquired. Separation strategies can therefore be goal-oriented by design. Detailed information on possible approaches towards either information or material can be seen in table 1.

Table 1. Development of separation strategies.[3]

"Classical" (by evolution)	**"Goal – oriented"** (by design)	
	Goal of separation	
Size based • Column size • Particle size of packing • Sample size	Information *Peak chromatography* For fastest information generation	Material *Product chromatography* For best product recovery
unstructured	Optimize for: • Separation sensitivity • Peak capacity • Speed • Selectivity	Optimize for: • Purity • Speed • Productivity • Eluent consumption

The types of chromatography can be distinguished by the bed shape (column-, thin layer-, paper chromatography etc.), physical state of the mobile phase (gas, liquid and supercritical fluid chromatography) and separation mechanisms (ion exchange, size exclusion, reversed phase, affinity, chiral chromatography, etc.).[4]

2.2 Electrochromatography

As the whole project´s goal is to manufacture an efficient continuous annular electro chromatograph, the stationary phase is tested via capillary electrochromatography. Electrochromatography is a chemical separation technique in analytical chemistry, biochemistry and molecular biology used to separate mostly large biomolecules such as proteins.[5] Recent trends in the pharmaceutical industry try to implement the electrochromatography as a continuous purification step into their process line. The combination of both size exclusion chromatography and gel electrophoresis results in a very fast and efficient separation of basic chemicals and active pharmaceutical ingredients (APIs). The stationary phase is required to have a specific micro and mesoporose structure, for allowing high separation efficiency and a high troughput. Additionally, the electroosmotic flow (EOF) through the stationary phase has to be adequate and constant. The basic mechanism in electrochromatography is the separation due to the analytes size. Molecules get trapped between/into orifices of the gel column thus prolonging their retention time. The meso- and microporous structure of the stationary phase influences which molecular size range is affected. Additionally, the applied voltage accelerates compounds with high electrophoretic mobility, providing the second separation mechanism. This mechanism is very important for separating compounds of nearly the same size and partition coefficient (e.g. amino acids).[6]

Furthermore, there are other factors influencing the chromatographic separation like the dimension and construction of the chromatographic cell (column), the voltage and the current applied, the properties of the solvent plus the possible reactions on the electrodes, the concentration and electrical properties of the ions, the temperature, influencing the movement of ions, the adsorbability of ions and the possible formation of complexes by the ionic mixture with the solvent.[7],[8]

2.3 Annular Chromatography

The continuous annular chromatography (CAC) is a continuous chromatographic method, which allows the separation of multi-component mixtures as well as of bi-component ones. In CAC, the mobile and stationary phases move in a crosscurrent fashion, transforming the typical one-dimensional batch column separation into a continuous two-dimensional one.[9]

Figure 1 outlines the basic principle behind the annular set-up of a CAC. The feed is introduced in a continuous manner at a fixed inlet point on top of the chromatograph. The cylinder with the stationary phase is slowly rotating. Additionally, eluent is constantly flushed to the column from top. After a given time (due to the chromatographic mechanisms pointed out in point 3.4.) the separated components of the feed appear at the bottom of the stationary phase. Chromatographic separation only takes place in the axial dimension. Depending on the retention time of the substances in the stationary phase, assuming constant rotation speed and constant separation constants over the whole stationary phase, certain compounds will always appear at a certain angle in relation to the inlet point. This allows a continuous process with the goal of purification of pharmaceutical compounds or separation of basic chemicals.

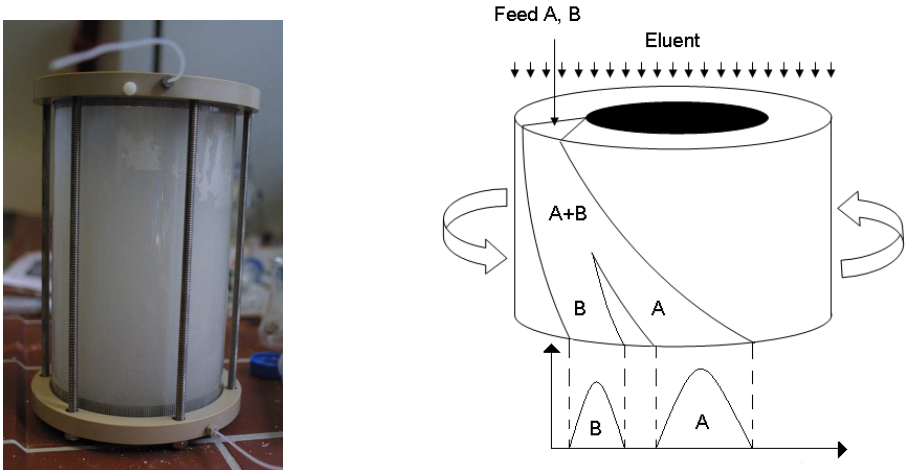


Figure 1. left: Annular filling frame; right: Scheme of an anullar chromatographic operation.[10]

3 Background

3.1 History of Chromatography

First attempts of using chromatography dealt with the separation of plant pigments and dyes, namely chlorophyll and carotenoids. In the 1860´s Christian F. Schönbein and his student Friedrich Goppelsroeder studied the different rates at which substances passed trough ordinary filter paper. Schönbein called this technique capillary analysis in the (false) assumption that capillary forces rather than adsorption were responsible for the separation.[11]
Also Schönbein used the dissolved analyte as eluent, creating overlapping zones rather than distinct points or bands created by modern paper chromatography.

Schönbeins method of capillary analysis was carried out without much change till the 20th century.

In 1927 Raphael E. Liesegang began placing filter strips in closed containers, which atmospheres were saturated by solvents.

In 1943 he refined his method by using discrete spots of sample adsorbed to the filter paper and dipped the paper into pure solvent to separate his test compounds.

This approach was published just before the far more influential work of Archer Martin et.al, which marked the beginning of the propagation of paper chromatography.

As for column chromatography the Russian botanist Mikhail Tsvet noted that the separation of samples via filter paper extraction can be applied to the methods of column fractionation, developed in the 1890´s for separating petroleum into its compounds. He was the first one using a liquid adsorption column packed with calcium carbonate to separate plant pigments.[3] This method was described on December 30th 1901 at the 11th Congress of Naturalists and Doctors in Russia, St. Petersburg.

The first written papers about his research were published in 1903, first mentioning the term chromatography in two of his works in 1906.

Additionally, he described the extraction of different samples off a filter paper. He noticed that carotene can be extracted by non-polar solvents while chlorophyll required polar solvents. This was a first step for understanding the basic principles of chromatography, the partition of different compounds in different medias.

Archer John Porter Martin and Richard Laurence Millington Synge merged the chromatography technique with countercurrent solvent extraction, revolutionizing the field of separation chromatography by being the first to separate chemicals with only minimal differences in their partition coefficients between two liquids. After a more or less failed experiment with complex countercurrent extraction machines and liquid - liquid chromatography, were the liquids moved in opposit directions, they developed a method of separation through a silica gel column, holding water stationary with a organic mobile phase. First the effectiveness of this technique was demonstrated by separating amino acids marked by methyl red.[12] After that they described several methods of separating organic chemicals in publications, starting with 1941.

But Martin and Synge not only focused on column chromatography, they also developed methods of separating amino acids by paper chromatography in 1944.[13]

Fred Sanger´s success of determining the peptide sequence of insuline was hugly based on Martins and Synge´s work on investigating the pentapeptide sequence of Gramicin S.[14]

In 1952, during his lecture for winning the Nobel Prize in Chemistry (shared with Synge), Martin noted that he and Anthony T. James successfully separated a wide variety of natural compounds via their developed gas chromatography.[15]

The easy and efficient appliance of gas chromatography encouraged the rapid adoption and development around the world. One main problem, the detection of substances at the end of the separating unit inspired N.H. Ray in 1954 developing the thermal conductivity detector, a foundation stone for J.Harley, W. Nel and V Pretorius´s flame inonization detector (1958).

In the same year 1958 James Lovelock descriped another new approach for detecting substances, the electron capture detector.[16]

In the 1960 HPLC (High performance liquid chromatography) was investigated, enhancing the speed of chromatographic analyses and thus making it interesting for industrial purposes.[16]

One of the most recent development was in 1987 by Pedro Cuatrecasas and Meir Wilchek, who also were awarded with the Wolf Prize in Medicine for their research in the field of affinity chromatography.[18]

Recognizing the fact that HPLC has limitations in efficiency due to the decreasing size of the packed particle where back pressure gets too high eventually, Capillary Electrochromatography has emerged as a hybrid between pressure-driven and capillary electrophoresis over the last 2 decades.

3.2 History of Electrochromatography (EC)

In the late 1980s a new chromatographic separation technique took the step from a research technique to a routine technology in many industrial environments.[5]

The possibility of reaching high separation by application of voltage, plus the benefits over HPLC, namely reduced method development time, reduced operating costs and solvent consumption and higher separation efficiencies opened new approaches, especially for pharmaceutical companies to purify their APIs. The biggest disadvantages of EC and CEC are mainly instrument related, like poor injection precision and sensitivity.

In 1939 Strain used a Tswett adsorption column to separate pigments by applying an electrical potential, this was the first use of EC despite that the word electrochromatography was first mentioned in 1943 by Berraz.[19],[20],[21]
EC was first mentioned in an article by Pretorius et al (1974), showing that moving the mobile phase trough a column via electroosmosis instead with the use of a pump, gave lower plate heights and thus a higher plate number of the column.[22]

3.3 History of Capillary Electrochromatography (CEC)

In 1981 Jorgenson and Lukacs adopted the approach of Petorius and filled capillaries with an inner diameter of 170 µm with octadecylsilica (C18) particles.[23] Practical difficulties and little improvements compared to regular liquid chromatography using pressure anticipated the breaktrough of CEC. Despite the poor packing procedure, they recorded, however, that the column had a surprisingly high efficiency, indicating that the electroosmotic flow (EOF) is uniform and not dependent on inhomogeneous packing.

3.4 Theory of Chromatography

As mentioned in the introduction, the separation mechanism of chromatography is based on the different partition coefficients of different compounds within a mixture. As for the terminology in our case of liquid chromatography the stationary phase is the solid, immobilized (silica) matter on the capillary wall. The mobile phase is described as the liquid solvent (also referred as eluent) solubilising the analyte and passing through the stationary phase. As for explaining the mechanism of adsorption, the adsorptives (free state) get immobilized by the adsorbent (stationary phase) and are described as adsorpt while bounded.
The separation can be divided into 3 steps.[3]

- In the beginning a second phase is formed (through introduction of thermal energy or as in case of adsorption, through an additive) in addition to the initially homogenous mixture.
- Thermodynamic imbalance between these two phases is the driving force for the mass and heat transport.
- After the exchanging procedures, the two phases possess different concentrations and are separated.

The main reason for different retention times of chemical compounds rests upon the adsorption of the analyte.

In fig 2 the basic mechanism of separation in chromatography, namely the different retention times of compounds in the column through attractive forces, is depicted.

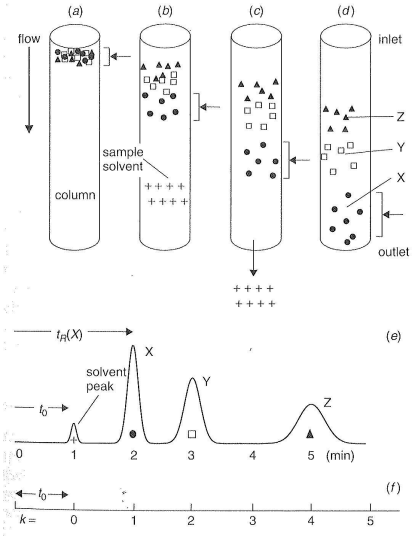


Figure 2. Principle of chromatography.[17]

There are two adsorption mechanisms:

- The chemisorption, driven by chemical reactions on the surface of the stationary phase with the adsorptive. This strong interaction results in ionic or covalent bonds, adsorption enthalpies ranging from 60 to 450 kJ*mol^{-1}.[3] The chemisorption is characterized by a high enthalpy change (exothermic reaction) and an activation energy for the reaction. This adsorption mechanism only takes place in a monolayer.
- The physisorption instead is relatively weak compared to the chemical adsoption. It is induced by a dipole moment resulting in so called Van der Waals forces with adsorption enthalpies of max 50 kJ*mol^{-1}.[3] Unlike the chemisorption the molecule adsorbed maintains its chemical identity. Also desorption processes are much more likely.

In chromatography molecules want to be recovered from the column. Thus, physical adsorption mechanisms are much more relevant. Stationary and mobile phase are adapted to meet the requirements for the best separation through different retention times of different compounds.

In general different separation problems require specific separation mechanisms to yield the best results. Another important role the detector plays, again there are better and worse choices for a separation problem.

3.5 Theory of Capillary Electrochromatography (CEC)

CEC combines both miniaturization, efficiency and resolution of capillary electrophoresis (CE) with the high selectivity and universality of liquid chromatography (LC).[24]

The driving force in CEC is the electroosmotic flow (EOF) forcing the mobile phase to move through a stationary phase. In contrast to CE where the surface charge on the capillary wall is responsible of maintaining an EOF, in CEC the particles constribute most to the EOF.[25]

The flow velocity can be calculated with the Smoluchowski equation where ε_0 is the permittivity of the vacuum, ε_r the mobile phases dielectric constant, ζ the zeta potential which is dependent on the electrical double layer δ, E the electric field and η the viscosity of the mobile phase.

$$\vartheta_{l0} = \frac{\varepsilon_0 * \varepsilon_r * \zeta * E}{\eta} \qquad \textbf{Equation 1}$$

The number of functionalized (chargeable) groups on the stationary phase is important for the EOF, also the purity, surface area and pore size of the stationary phase. In most (industrial) cases, silica is used as stationary phase, functionalized with silanol groups. Furthermore the silica backbone can be functionalized e.g. with alkyl groups.

The EOF can additionally be controlled by the composition, pH, ionic strength and organic modifiers of the mobile phase.

As it can be seen in fig.3, the flow profile of pressure driven systems is parabolic. In contrast, the flow profile of the EOF is flat, because it is induced at the interface between particle - liquid and capillary wall-liquid. Electrical double-layer overlap does not occur as long the spare distance between particles doesn't exceed 20 times δ.

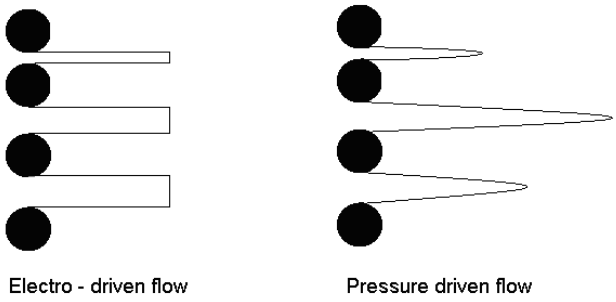


Figure 3. Flow profiles of an elctro - driven flow (left) and a pressure driven flow (right).

This double-layer overlap has negative effects in CEC because (i) it reduces the electroosmotic velocity and (ii) a double layer overlap causes a peak dispersion through a transition of the flat flow profile into a parabolic one.[26]

Regarding the efficiency, CEC is able to achieve a much higher one than LC. The lower plate heights of the CEC are attributed to the flatter flow profile. Additionally, the flow variation in the stationary phase is smaller, thus less Eddy diffusion. Also the particles do not hinder the flow, rather they propel the liquid trough the stationary phase because the EOF originates at the particle surface.[25] In CEC it is also possible to use smaller particles than in HPLC (no backpressure). Looking at the Van Deemter equation (equation 11) both A and C terms decrease with smaller diameter of the particles used, lowering the theoretical plate height N (see 3.6).

3.6 Parameters of Chromatography

The main parameters of chromatography are efficiency, resolution and selectivity.

Efficiency:

To characterize the efficiency of the column Martin and Stringe introduced the term of the theoretical plate height.[27] N, the plate number characterizes the theoretical separation steps, meaning how often a thermodynamic equilibrium is achieved within a column. Typically numbers of plates range between 1.000 and 10.000. The length of the column and packing procedures as well as the properties of the stationary phase have an impact on the theoretical plate height number H.

Resolution:

The resolution gives information of the discreetness of two consecutive peaks. If both areas of both peaks can be measured accurately a good resolution is given. Parameters influencing the resolution are the same as the ones affecting efficiency and selectivity.

Selectivity:

The selectivity is closely linked to the partition coefficient, it describes the ratio of molecules in the mobile phase to the molecules in the stationary phase.[16]
Selectivity can be influenced by temperature, ph-value, type and concentration of solvent and type of packed column.[28]

Given the fact that the separation efficiency in a chromatographic column is based on the partition and the thermodynamic equilibrium, the equilibrium constant K (equation 2) and the phase ratio Φ (equation 3) are introduced.

$$K = \frac{c_s}{c_m}$$ **Equation 2**

c_s…concentration of molecules in the stationary phase
c_m…concentration of molecules in the mobile phase

$$\phi = \frac{V_s}{V_m}$$ **Equation 3**

V_s… Volume of the stationary phase in a column
V_m… Volume of the mobile phase in a column

Information about the chromatographic separation process can directly be obtained through a chromatogram.

Figure 4 shows a typical chromatogram of a sample with three different molecules. The interaction of each molecule or component of the sample mixture with the stationary phase is proportional with its retention time t_R.

Unusual but also possible is the use of the retention Volume V_R which is calculated by multiplying t_R with the flow rate. The retention time of a molecule is derived by its peak maximum in case of a symmetrical peak.

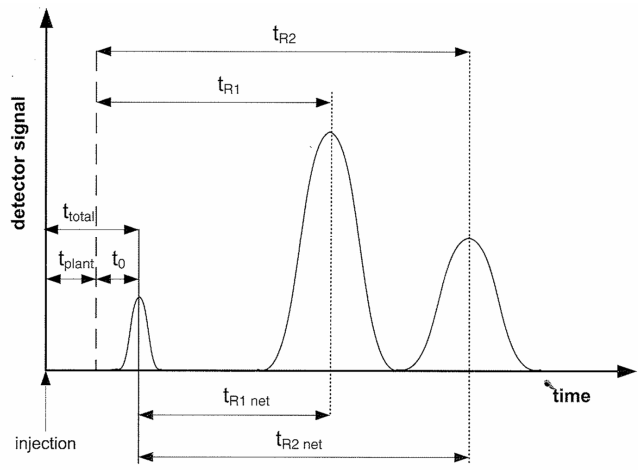


Figure 4. Chromatogram of three different molecules.[3]

Table 2. Parameters of a chromatogram.

Parameter	Description
t_{plant}	Dead time of the plant without the column
t_0	Dead time of the column
t_{total}	Total dead time
t_{R1}, t_{R2}	Overall retention time of the components 1, 2
$t_{R1\ net}$, $t_{R2\ net}$	Net retention time

The total dead time t_{total} is the time a non retained molecule needs from the point of the injection to the detector. This parameter depends on the hold up in the column as well as of the hold up in the whole system or plant. The hold up in the plant is influenced by pipe diameters and lengths, the column, pump head volume and detector volume.

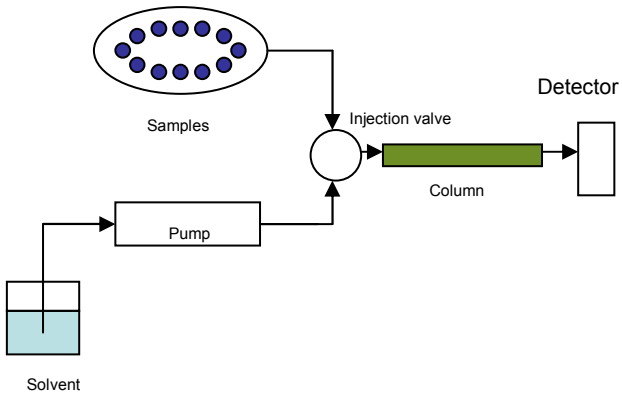


Figure 5. Instrumentation of a typical HPLC Plant.

Therefore, both the dead time of the plant without the column t_{plant} and the dead time of the column t_0 have to be determined. ($t_{total} - t_{plant} = t_0$).
For this purpose tracer molecules are introduced with the analyzed sample. These tracers should not interfere with the analyte(s) and the stationary phase, but should have about the same size as the molecules analyzed to prevent size exclusion separation phenomena.

The overall retention time $t_{R,i}$ of the retained molecule *i,* assembles out of the dead time of the column t_0 and the net retention time $t_{R,i\ net}$.

Due to the fact that the net retention time describes the time of the molecule adsorbed on the stationary phase and the dead time of the column is dependant on the tracer used to determine it, the calculation of the net time is rather difficult. Therefore, the overall retention time $t_{R,\ i}$ is the international established value for describing the retention of a compound in liquid chromatography.

Of course the retention time of a substance is also influenced by the mobile phase flow rate and the geometry of the column. Thus the capacity factor k', also called the retention factor, is defined as follows:

$$k' = \frac{(t_{R,i} - t_0)}{t_0} = \frac{n_{stat,i}}{n_{mob,i}} \qquad \textbf{Equation 4}$$

k' depends on the distribution of the component between stationary and mobile phase (the equilibrium constant) and describes the adsorption time in relation to the time in the mobile phase. This can also be expressed as the mole (n_i) ratio of the compound *i* in the stationary ($n_{stat,\ i}$) and mobile phase ($n_{mob,\ i}$).

Given this parameter, the selectivity α (= separation factor) of a column separation can be calculated.

$$\alpha = \frac{k'_1}{k'_2} = \frac{(t_{R,1} - t_0)}{(t_{R,2} - t_0)}$$ **Equation 5**

The more retained component is always the denominator, thus, the selectivity is always > 1.

For describing the efficiency of a column in chromatography Martin and Synge introduced the terms plate number N and plate height H. Theory behind this is that on every plate a thermodynamic equilibrium between stationary and mobile phase is achieved. These parameters were merged in the height of an equivalent theoretical plate (HETP) equation for a column with the length L_c.

$$HEPT = \frac{L_C}{N}$$ **Equation 6**

For uniform packed columns, incompressible eluents and the inclusion of the standard deviation σ_t of the time dependent peak of the analyte, the plate height can be calculated.

$$HEPT = \left(\frac{\sigma_t}{t_R}\right)^2 * L_C$$ **Equation 7**

The higher the efficieny of a chromatographic column the more rectangular (=ideal) and narrower the peak appears in the chromatogram. This “ideality” is however limited by the mass transfer and fluid dynamics, so that every peak has a limited efficiency.

The plate number N_i can also be derived out of the chromatogram.

$$N_i = \left(\frac{t_R}{\sigma_t}\right)^2 = 16 * \left(\frac{t_{R,i}}{w_i}\right)^2$$ **Equation 8**

Another approach uses the peak width at half height $w_{1/2,\,i}$.

$$N_i = 5.54 * \left(\frac{t_{R,i}}{w_{1/2,i}}\right)^2$$ **Equation 9**

To compare columns of different dimension eq.10 can be used

$$N_{L,i} = \frac{N_i}{L} = \frac{1}{HETP}$$ **Equation 10**

All these equations apply for symmetrical peaks, for asymmetric peaks please check the according literature.[3]

The Van Deemter equation takes variances per unit length due to pathways within the column, diffusion (axial and longitudinal), and mass transfer kinetics between stationary and mobile phases into account. The simplified equation 11 does not take the mutual influence of the parameters themselves into account. For example when two inter-particle flows stream together, they combine and remixing occurs, that would mean a loss in the velocity profile. This simplified equation was chosen to show up the possible imperfections of the manufactured column.

$$H_i = A_i + B_i * u_{\text{int}} + \frac{C_i}{u_{\text{int}}}$$ **Equation 11**

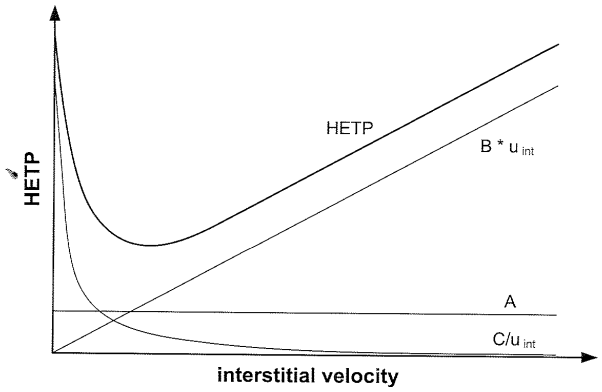


Figure 6. Parameters of the Van Deemter equation.[3]

The *A Term* derives from the so called eddy diffusion (fig 7.c), resulting from packing imperfections and / or stationary phases with very broad particle size distribution. By using smaller particles of a more uniform PSD (particle size distribution) the plate height can be decreased.

$$A = 2 * d_P * \lambda$$ **Equation 12**

λ takes the particle size range, the column geometry and dimensions plus the packing uniformity into account. d_p is the diameter of the particle.

The *B term* describes the increase of the plate height at high velocities of the mobile phase, aggravating the reaching of the equilibrium. Mass transfer

resistance increases at higher velocities (interstitial velocity u_{int}), this influence can be reduced by optimising pore sizes (where the mass transfer resistance is nearly independent on fluid velocity) and diffusion pathlength.

The term for longitudinal diffusion, describing the axial movement of the molecules parallel to the flow of the mobile phase, is expressed by *term C*.

The lower the flow rates and the higher the particle size of the stationary phase, the more this term increases the plate height. Therefore, a rather high flow rate of the mobile phase and the use of a low viscosity solvent is preferred, bearing in mind that the pressure drops of the column is also lower.

In fig 7 and fig 8 the different imperfections in a column and the consequent fluid distribution non - idealities are pointed out.

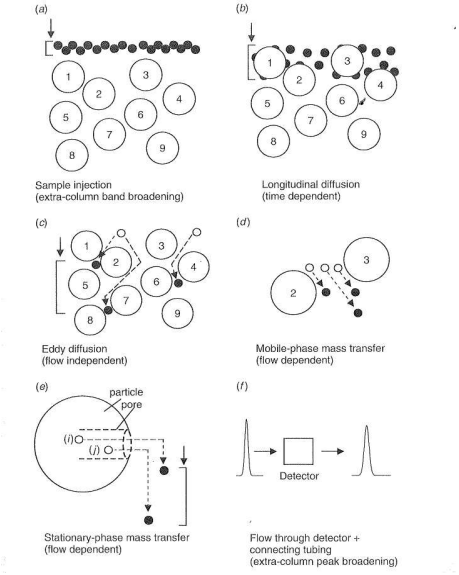


Figure 7. Various contributions to band broadening in LC.[17]

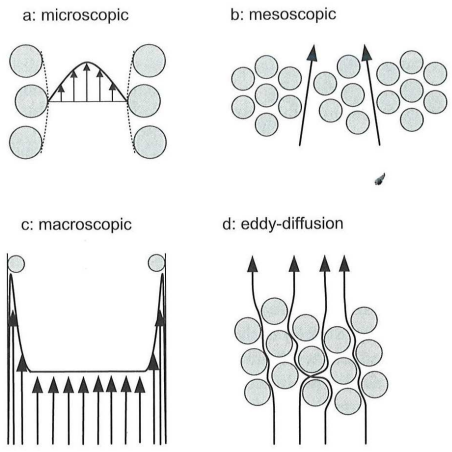


Figure 8. Fluid – distribution non – idealities according to Tsotsas (1987).[3]

To outline the conditions for good separation, meaning a low theoretical plate height, also described by a low minimum of the Van Deemter curve:

- Small particle size (and distribution) for the stationary phase.
- Small column diameter.
- Homogeneous packing.
- Mobile phase of low viscosity and a high flow rate inducing small diffusion coefficients in the mobile phase.
- Stationary phase with adequate pore size for the separation problem, inducing high diffusion coefficients in the stationary phase.
- Optimizing thermodynamic parameters as the temperature of the system.

Especially preparative chromatography requires complete peak resolution, guaranteeing that the compounds separated are 100% in yield und purity.
The chromatographic resolution parameter R_S describes how discrete two adjacent peaks really are. Resolution, as already mentioned in eq. 14 and 15, depends on selectivity, capacity factors and efficiency.

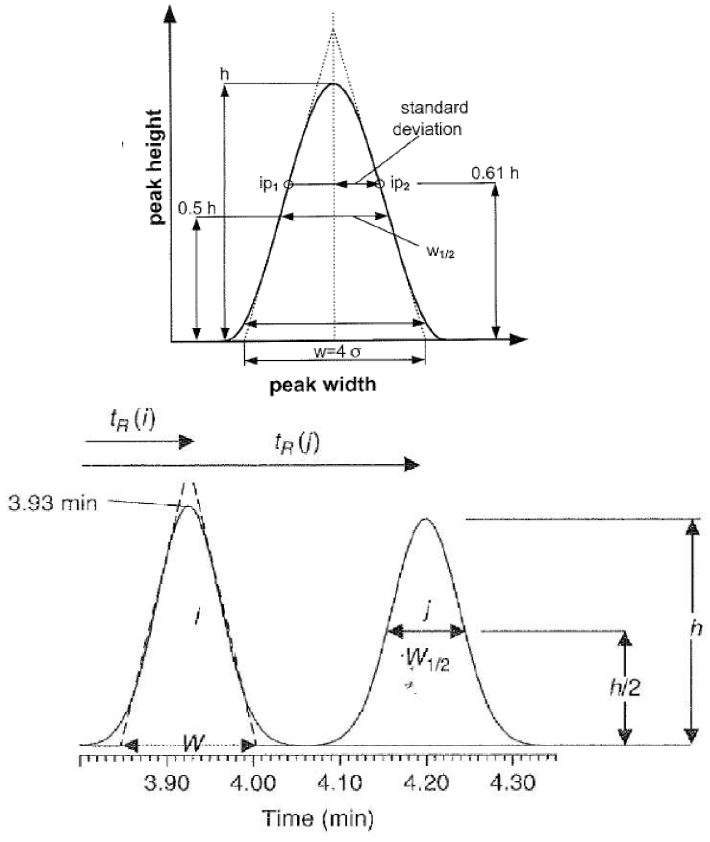


Figure 9. Resolution parameters.[3],[17]

$$R_S = 2 * \frac{(t_{R2} - t_{R1})}{(w_1 + w_2)}$$ **Equation 13**

w_1 and w_2 are the peak widths at the base line of the particular peak. Assuming equivalent peaks, where $w_1 = w_2$

$$R_S = \frac{(\alpha - 1)}{\alpha} * \frac{k'_2}{(k'_2 + 1)} * \sqrt{\frac{N_2}{4}}$$ **Equation 14**

Or for the less retained component.

$$R_S = (\alpha - 1) * \frac{k'_1}{(k'_1 + 1)} * \sqrt{\frac{N_1}{4}}$$ **Equation 15**

3.7 Porosity

The porosity as well as the specific surface area of stationary phases are two parameters which are often used to determine the quality of a manufactured column.

In the stationary phase we can characterize different volumes.[3] The total volume of the column V_{total} summarizes the interstitial volume of the fluid phase V_{int} and the volume of the stationary phase V_{stat}. Given a cylindrical shape of the column, where L_C is the lengh and d_C is the diameter of the column, the total volume can be depicted as

$$V_{total} = \pi * \frac{d_C}{4} * L_C = V_{\text{int}} + V_{stat}$$

Further the volume of the stationary phase V_{stat} can be divided into the volume of the solid material V_{solid} and the volume of the pores V_{pore}.

$$V_{stat} = V_{solid} + V_{pore}$$

These volumes set the basis for further calculations of different porosities.

$$\varepsilon = \frac{V_{\text{int}}}{V_{total}}$$ Void fraction

$$\varepsilon_{solid} = \frac{V_{pore}}{V_{stat}}$$ Porosity of the solid phase

$$\varepsilon_{total} = \frac{V_{pore} + V_{\text{int}}}{V_{total}} = \varepsilon + (1-\varepsilon) * \varepsilon_{solid}$$ total porosity

According to literature the porosity of silica based monoliths is about 80%.[17]

3.8 Detectors

In the simplest way possible, as for the separation of dyes or coloured plant pigments for example, the human eye can act as detector. As for modern liquid chromatography (LC) several detectors were established. One major disadvantage of LC in comparison to gas chromatography (GC) is that no detector of the LC compartment has an equivalent performance to the GC´s flame ionization detector (FID). In general, LC detectors have a sensitivity 2 - 3 orders of magnitude less than the FID.[4]

Therefore, several detectors were invented, aiming for the highest sensitivity in a rather small field of chemical compounds.

The most important and widely used ones (making up to 80% of total chromatographic analysis) are described below.[17],[29]

3.8.1 The Electrochemical Detector

This type of detector includes electrodes and responds to oxidable or reducible substances, thus, the detection mechanism is the electron flow generated by the redox - reaction at the surface of the electrodes. If the reaction proceeds completely (meaning all of the reactants are consumed) the electron flow (equals the current) becomes zero, now the total charge is proportional to the total mass of substances which reacted. This method is called coulometric detection.

The second possible method, the amperometric detection, takes into account that the reactant is continuously replaced as the peak passes throught the detector, thus a current is maintained all the time, although varying in magnitude.

Both detection modes require three electrodes. The working electrode (where redox-reactions take place), the reference electrode (compensates background conductivity of the mobile phase) and the auxiliary electrode.

The thermodynamic explanation for this detector is a concentration gradient. Near the electrode reactants are consumed due to redox-reaction. Therefore, the concentration of reactants near the electrode is lower than in the bulk of surrounding mobile phase.

The concentration gradient forces diffusion proportional to the solute concentration of the mobile phase. So the whole detection mechanism is diffusion controlled, dependent on reactant concentration and diffusivity.

The electrochemical detector is especially used in industry when it is necessary to detect neurotransmitters.

3.8.2 The Fluorescence Detector

Fluorescence describes the process when an orbital electron of a molecule or atom relaxes to its ground state by emitting a photon after being excited to a higher quantum state by energy – input. It is a very sensitive detector able to detect naturally fluorescing compounds like Riboflavin (Vitamin B2) in the pg range.[31]

The adsorption of light is highly specific for the molecules or atoms affected. Wavelength or energy absorbed is dependent on particular molecular structure respectively possible quantum single states.

Fluorescence detection techniques are the opposite of light absorption measurements and focus on the detection of the fluorescent light emitted by the sample.

3.8.3 The UV Absorption Detectors

This type of detector is the most widely used. Many chemical substances absorb light in the range of 180 to 350nm (range of UV - light, just below the Visible Light (VIS) range). The mechanism of detection is based on the relationship of the original light intensity directed through a measurement cell with exact measurements, containing a certain concentration of analyte, to the light intensity transmitted. This relation is described by Beers Law

$$E_\lambda = -\log\left(\frac{I}{I_0}\right) = \varepsilon_\lambda * c * d \qquad \textbf{Equation 16}$$

Where E_λ is the extinction at the specific wavelength λ, I is the intensity of the transmitted light while I_0 is the intensity of the incident light, ε_λ describes the extinction coefficient at a specific wavelength *λ*, *c* the concentration of the sample analyzed and *d* the film thickness of the sample.

3.8.4 LC Detectors Based on Refractive Index Measurement

Refraction is a phenomenon of scattered light. If a beam of light is directed from one phase to a second phase with different properties it alters the wavelength (velocity) of the light wave as well as the main angle of the light beam.

The refraction index is highly dependent on both the mobile phase used and the temperature.

Taking into account that every molecule has its own refraction index, this technique can be applied to a wide field of detection problems but they severely lack sensitivity.

In table 3 the most commonly used detectors and applications in industry are summarized.

Table 3. LC - detectors and their industrial application.

Detector	Molecule properties	Detectable substances
Electrochemical Detector	Oxidable or reductable/ionic molecules	neurotransmitters
Fluorescence Detector	Fluorescent molecules	anthracene, stilbene, toluene…
UV Absorption Detector	Double bonds/aromatic, unshared electrons	aromatics, unsaturated olefines, carboxyl groups...
RI-Detector	Not specific	general use

3.9 Separation Mechanisms

During the development of liquid chromatography, several techniques of separation were established, the most important are partition chromatography, ion chromatography, size exclusion chromatography and surface adsorption chromatography.[16],[31],[32]

Table 4. Separation mechanisms.

Chromatographic method	Based on
Surface Adsorption Chromatography	Van der Waals forces
Partition Chromatography	Partition coefficient
Ion Exchange Chromatography (IEC)	Ionic strengh
Size Exclusion Chromatography (SEC)	Size / molecular weight (proteins)

3.9.1 Surface Adsorption Chromatography

Driving force behind this separation method is the different polarity of feed components respectively Van der Waals forces. The more polar the molecule, the stronger it will be adsorbed and retained by a polar stationary phase. Same applies to a non - polar molecule and a non - polar stationary phase. Feed molecules with the same polarity as the stationary phase get delayed stronger on the way through than molecules of opposite polarity. Additionally, the strength of

the dipole moment of the sample molecules (ranging from nonexistent – non - polar to strong – polar substances) influences their specific retention time.

Stationary and mobile phase have to be adequate to the separation task. The most common and widely used adsorbents in liquid chromatography are porous alumina and porous silica (gel).

3.9.2 Partition Chromatography

Due to the very low diffusion rates in solids, partition chromatography uses a liquid stationary phase, immobilized (coated) via a covalent bond onto a solid carrier such as silica gel, cellulose or hydrated silica. Assuming there is no adsorption by the solid support, the sample molecules retention time is dependent only by the partition / solubility between the liquid mobile and the liquid stationary phase. There are two different techniques of partition chromatography. The normal phase chromatography is characterized by the use of a hydrophilic stationary phase and a hydrophobic mobile phase and the reversed phase chromatography with hydrophobic stationary and hydrophilic mobile phase.

3.9.3 Ion Exchange Chromatography (IEC)

The basic principle of IEC is the use of charged substituents on ligands immobilized onto a porous resin. When the mobile phase passes by, the sample molecules (which have to be counter - charged / ionised to the stationary phase) get loosly complexed with the charged substituents of the stationary phase. The stronger the affinity between the feed ions and stationary phase, the higher the retention times.
Most widely used are stationary phases made of synthetic copolymer of styrene and divinyl benzene (DVB). With the modification of the DVB concentration the degree of cross linking and hence the porosity of the resin can be influenced.

The produced resin has to be chemically modified to either fit cation (+) or anion (-) exchange applications.

3.9.4 Size Exclusion Chromatography (SEC)

SEC (also gel permeation chromatography) uses a porous polymeric gel. The size and size distribution of meso- and micropores determine the size range of molecules just passing the pores with the mobile phase. Smaller molecules diffuse into the pores instead and hence will be more retarded than bigger molecules. Therefore compounds elute in order of size or molecular weight.

3.10 The Sol–Gel Process

Beginning in the mid 1800s with Ebelman and Graham sol-gel processes caught attention.[33],[34],[35],[36] They noted, that the hydrolysis of tetraethyl orthosilicate (TEOS, $Si(OC_2H_5)_4$ yielded SiO_2 particles under acidic catalysis. These “glass-like” matter, as they described it could be drawn into fibers and monolithic lenses. The main problem of extremely long drying times (over 1 year) prevented industrial interest and thus further development. In the 1950s and 1960s Roy and co-workers saw the potential of this process to produce very high homogeneity colloidal gels.[37],[38],[39] Roughly the same time Iler worked on the field of silica chemistry making colloidal silica powders commercially available.[40] Stober et al. refined Iler´s approach, using ammonia as catalyst for the TEOS hydrolysis, also controlling both morphology and powder particle size through varying the content of ammonia, to yield the widely known spherical Stober particles. In his work Stober mentioned that the final size of spherical silica powder is dependent on the initial concentration of water and ammonia, the type of silicon alkoxide and alcohol used.[41] Further the reactant temperature has a huge impact on the morphology.

According to literature there are three approaches of making sol gel monoliths.[33]

1. Through gelation of a solution of colloidal powders
2. Hydrolysis and polycondensation of alkoxide precursors followed by hypercritical drying
3. Hydrolysis and polycondensation of alkoxide precursors followed by aging and drying under atmospheric conditions

These monoliths are in contrast to discrete particles integral.

As for the terminology: Sols are described as dispersions of colloidal particles in a liquid. A gel is a rigid and interconnected network with pores of different dimensions (micro-, meso-, macropores) and polymeric chains.

A silica gel can thus be formed by an array of discrete colloidal particles (method 1) or by the formation of a 3-D network by hydrolysis and polycondensation of organometallic precursors (method 2 and 3). One main problem (especially for manufacturing silica monoliths) is shrinkage. Shrinkage occurs when the pore liquid is evaporated under atmospheric conditions producing cracks in the monolith due to missing capillary forces. Detailed mechanisms of syneresis and the consequences are explained later. The method of choice in this thesis was hydrolysis and polycondensation of alkoxide precursors followed by aging and drying under atmospheric conditions. The sol - gel process is thus divided into hydrolysis, condensation, aging and drying.

3.11 Hydrolysis and Condensation

Basically three reactions occur during a sol gel process, namely hydrolysis, water condensation and alcohol condensation.

Hydrolysis happens when the precursors are mixed together in either an acidic or basic environment, which catalyzes the hydrolysis reaction.[42] This process can be accelerated dramatically by raising the temperature. Hydrolysis yield hydroxyl groups at the monomers (tetraethoxysilanes), these triethoxysilanoles then polymerize either through water or alcohol condensation to the final product. Triehtoxysilanoles are highly reactive, so condensation reaction can happen during hydrolysis. Basic mechanisms of these reactions can be seen in fig 10 and 11. There is a model for hydrolysis with a pentavalent transition state in literature.[33] At basic conditions the OH-ion has a high nucleophilic power and attacks the Si-atom directly (containing the highest positive charge). At acidic conditions the proton is attracted by the O-atom of the methoxy group. The positive charge of the Si-atom increases because there is an electron shift from the Si-O bond to the O-atom. This positive charge induces the attack of the water molecule and the pentavalent transition state is formed.

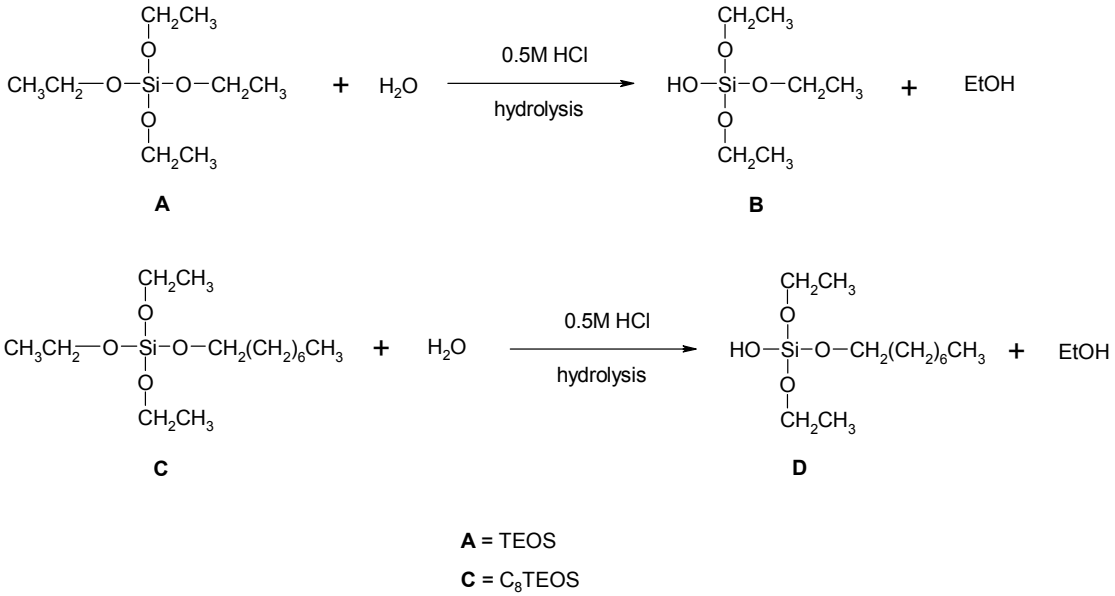


Figure 10. Hydrolysis of TEOS and C_8 TEOS.

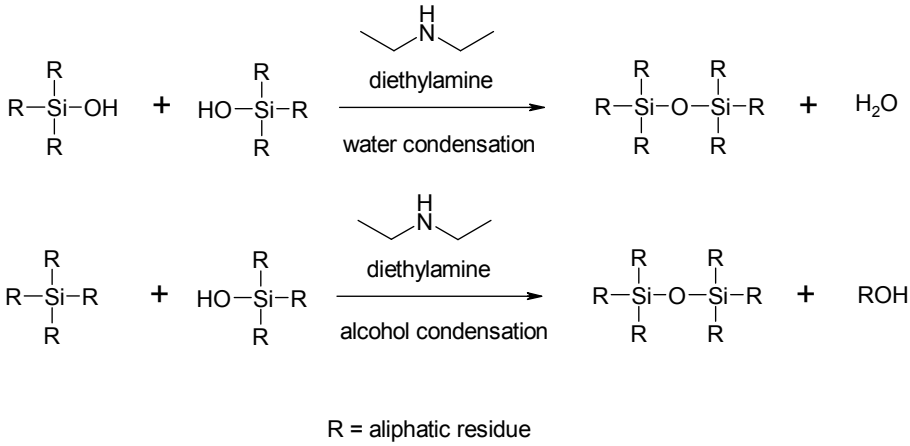


Figure 11. Principle of alcohol and water condensation reactions.

Table 5. Type of condensation reaction for compound combinations (A,B,C,D see fig.10)

Compound combination	Condensation reaction
A+B	Alcohol condensation
A+D	Alcohol condensation
B+C	Alcohol condensation
D+C	Alcohol condensation
B+D	Water condensation
B+B	Water condensation
D+D	Water condensation

In the pores of the gel network, the generated alcohol and water accumulates. The amount and ratio of these side products also determine pore size and pore size distribution during the drying process. In general all subsequent processes of aging and drying are influenced by the gel structure developed during the hydrolysis and condensation step.

3.11.1 Kinetics

As for hydrolysis and condensation there are many important factors, those with the most impact are temperature, nature and concentration of (acidic or basic) electrolyte, type and concentration of solvent and the type of alkoxide precursor. Also it is difficult to describe the process because hydrolysis and condensation take place simultaneously.
According to Hench and West, the temperature dependence expresses itself by a 10 fold increase of the hydrolysation rate of TEOS if the temperature ranges between 20 to 45.5°C. Further the choice of solvent influences the rate constant of hydrolysation k_h in the order by acetonitril > methanol > dimethylformamide > dioxane > formamide. Additionally to the ratio of water has an impact on the pore sizes, higher water content in relation to TEOS induces k_h to increase under acidic conditions.

However, the most important factor for controlling the hydrolysis rate is the electrolyte concentration and the steric hindrance of possible bulkier alkoxide groups of the precursors. As Hench and West report there is a 1500 fold increase of k_h by only a minute addition of HCl, also pointing out that the choice of the acidic catalyst plays a role in gelation time of TEOS.

Giving numbers for the rate constants of k_h and the rate constant of condensation k_c is difficult because as mentioned, there are several hydrolysis and condensation reaction happening simultaneously, each with its own time dependent rate constant. Generally as Hench mentions the presence of H_3O^+ ions increases hydrolysis rate whereas OH^- ions push the condensation.

In terms of polymerization / condensation characteristics, acidic conditions yield a more linear appearance of the linked monomers, while basic conditions boost the cross linking of monomers to more branched polymers. Reason for this, according to literature is the steric hindrance under acidic conditions.[33]

Also the ratio of k_h and k_c determine the appearance of the polymers. Fast hydrolysis and slow condensation favour more linear polymers while the opposite results in bulkier, ramified polymers.

3.12 Aging

The process of aging describes the ongoing changes in structure and properties long after the gelation point. Generally spoken a sol becomes a gel when it is able to support a stress elastically. There is a sharp increase in viscosity once this happens. There are four processes which can happen simultaneously or succedaneous, these are named polycondensation, syneresis (expulsion of the pore liquid), coursing and phase transformation.

During aging, referred to as syneresis, spontaneous shrinking of the gel can occur. Although polycondensation continues to strengthen the gel structure over time (if neighbouring silanols are close enough to react), the expulsed pore liquid due to the formation of bridging bonds and thus contraction of the network lead to decreasing surface tension of the solid / liquid interface and therefore a loss in stability of the gel network. Methanol prevents this to a certain extend by inhibiting condensation (lowering the rate constant k_c) by forming hydrogen bonds with the reacting silanols.

Coarsing is the surface area decrease through dissolution and reprecipitation. An aged gel has to possess a certain strength to resist cracking in the drying step. pH, temperature and solvent ratio are crucial in this phase for the emerging gel network. Especially the initial water content plus the produced water by hydrolysis have a dramatic effect on the gelation time (the less water the faster). Another parameter to consider is the molecule size, the bigger the precursor molecules, the slower gelation, the higher viscosity. Thus, viscosity is an important parameter if the emerging gel network wants to be brought into a 100 µm i.d capillary.

3.13 Drying

Hench and West reported three different stages of drying:

- In the initial phase of the drying process the decrease in total volume of the gel is equal to the loss of volume through evaporation of liquid. The network is deformed by capillary forces, causing shrinkage of the monolith. The end of Stage 1 is marked by the so called “leatherhard point”, the critical point in the drying process when the gel network has sufficient mechanical strength to resist further shrinkage.
- Stage 2 the pore liquid transport occurs by flowing to the surface where evaporation takes place. This liquid transport is caused by the capillary pressure caused by the densification of the material in stage 1. According to Hench and West stage 2 is the phase in the drying process where cracking of the monolith is most likely to occur.
- Stage 3 describes the evaporation of the remaining liquid by diffusion of vapour out of the pores to the surface.

Heat treatment has a beneficial effect on the stabilization process of the monolith by desorbing chemisorbed left over hydroxyl groups of the chemical reaction. Even though the desorbed hydroxyl groups decrease the contact angle and thus the sensitivity of the monolith to rehydration stresses, heat treatment in this temperature range causes the smallest pores to sinter, reducing number of fine pore and densifies the whole monolith.

Certain ratios of precursor(s), type and amount of solvent, porogens, catalyst plus certain hydrolysis, condensation and drying procedures determine the stability and porosity of the produced network so that shrinkage can be minimized and cracking prevented.[43]

3.14 Spinodal Decomposition

In a gel network there are two distinct phases. The (i) liquid phase enclosed by (ii) a solid phase. Theory behind this phase separation is the spinodal decomposition.[44]

Driving force of the spinodal decomposition is a difference in chemical composition and / or physical properties of compounds in a mixture. Unlike nucleation which only occurs at discrete nucleation sites, phase separation induced by spinodal decomposition occurs uniformly troughout the whole mixture. The whole process is diffusion controlled.

Spinodal decomposition is a mechanism for relatively slow dynamic materials. It starts with an infinitesimal composition fluctuation (with a characteristic wavelength), which increases in amplitude over time. The contrast between the two phase domains gets bigger and eventually a silica rich domain called silica skeleton or backbone and a solvent – rich domain (resulting in macropores) are formed.[44] These domains increase in size (coarsening) and finally result in a continous matrix with subdivided domains. Addtitives like water- soluble polymers or surfactants can be used to accelerate or induce phase separation. Polyethylenglycol (PEG) and *N,N,N*-trimethylhexadecyl - ammoniumbromide (CTAB) are widely used in sol gel processes and influence the volume and size distribution of macropores in gels.[42]

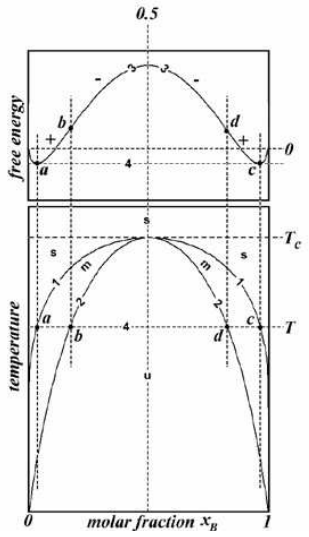


Figure 12. Diagram of free energy change and phase diagram.[46]

Fig. 12 shows a diagram of the free energy change (of mixing) (top) and a phase diagram with a miscibility gap (bottom). Line (1) is the phase boundary, above this line the system is stable which means the two liquids are miscible. Above the temperatue Tc, the liquids are miscible in any ratio. The (m) area marks the metastable region. Line (2) is the spinodal, below this line the system is unstable. The combination of region m and region u forms the miscibility gap. In this specific region of the diagram a one phase system turns into a two phase system. At a given temperature a line (4) cuts the phase boundary line (1) and the spinodal (2). The points a,b,d and c can be transferred into the free energy diagram above. There the segments ab and dc have a positive second derivative, segment bd a negative second derivative. A positive second derivative of the free energy of mixing (ΔG) applies to a stable (or metastable) system, a negative second derivative of ΔG points out an unstable system.

However, phase separation happens when the system is in the miscibility gap. In the metastable region the dominant mechanism of growth is nucleation, whereas in the unstable regions spinodal decomposition occurs.

3.15 Stationary Phases for CEC

Stationary phases can be inserted into CEC - capillaries in three different ways, each with its unique advantages and disadvantages.[3],[47]

3.15.1 Packed Columns / Capillaries

This type of packing is realized by filling particles of known size distribution into the column / capillary. There is a limit given through the size of the particles and thus emerging backpressure. Additionally, both ends of the column have to be sealed with frits to hold the content within. These retaining frits can lead to bubble formation in the operated column, decreasing the efficiency. Common packing material is silica - based or mixed mode, all of specified particle size distribution. For use in the CEC the stationary phase has to have ionizable ligands for generating an EOF. This is normally realized by using hypersil or octadecyl modified silica particles.

These two types of packing material have silanol groups, although the number is highly influenced by pH – levels. Mixed mode packing processes circumvent the influence of the pH by using ion exchangers.

Another disadvantage of this packing material is the rather complex packing process of columns, different approaches can be found in literature.[3]

3.15.2 Open Tubular Columns

With this method the stationary phase is coated to the inner capillary walls, there is no complete coverage through the whole diameter of the capillary.[48]

Stationary phases are either coated via dynamically or physically adsorbtion (or as it is called static coating). The dynamical method is characterized by a very weak bond to the capillary wall. It can be easily flushed out of the column with pure eluent, therefore the adsorbed stationary phase is also mixed to the eluent, creating a thermodynamical equilibrium between coated agent and mobile phase, thus preventing the desorbtion of the stationary phase.

The physically adsorption instead is considered rather strong. It is realized by flushing the empty column with (1N) NaOH, activating the inner surface of the capillary yielding Silanol (Si-OH) groups. These functional groups interact with the stationary phase via a covalent bound, fixing the stationary phase to the inner wall. Further substances like cationic and polymeric surfactants or charged polymers can be used to coat the inner wall of capillaries

3.15.3 Monolithic Columns

Beeing aware of the limitation of particle packed columns, namely the back pressure of tightly packed columns increasing with decreasing particle size, development of monolithic columns started. Yielding one single so called “rod” with an influence - able internal network of pores and polymer chains, the simple manufacturing procedure and possible functionalization of monolithic columns allow easy design for a specific separation problem. Basic mechanism for the polymerisation is the sol- gel process, explained in point 3.10.

Two possible starting precursors for monoliths are known, silica based and organic polymer based

Organic based monoliths:

Most widely used precursors for organic polymer based monoliths include methacrylate, styrene and acrylamide.[17],[47] The biggest disadvantage of these monoliths is the excessive shrinking due to the vaporized organic solvents during the aging and drying process, often resulting in cracking and / or swelling of the column.

Silica based monoliths:

Tanaka et al. describe in detail how silica based monoliths can be synthesized via different precursors. Mostly tetraethoxysilane (TEOS), tetramethoxysilane (TMOS) and octyltriethoxysilane (C8-TEOS) are used, all yielding a reversed phase column due to their apolar end groups. Silica monoliths can either be manufactured in a one or two step process.[49] Certain ratios of precursors, amount and type of solvent used, porogens (like CTAB and PEG,) and the aging and drying process influence the porosity and stability of silica based monolithic columns.

To summarize the advantages and disadvantages of the different types of stationary phases, especially in CEC, the characteristics of packed, open tubular and monolithic columns are compared in table 6.

Table 6. Characterisitcs of the different types of CEC - columns.

	Packed columns	**Open tubular columns**	**Monolithic columns**
Packing procedure	Packing process, retained trough frits	Packing process, retained trough frits	In situ polymerization Covalently bond to capillary wall
Stability	Low	Medium	High
Performance and Problems	Backpressure with decreasing particle size	Bubble formation, low capacity	High porosity, functionalization, shrinkage

4 Experimental

4.1 Activation

The goal of this thesis was the preparation of different silica based monolithic stationary phases that were implemented in CEC Capillaries. To chemically bound the stationary phase to the inner wall of the capillary, the capillary wall has to be “activated”. This is achieved by treating the fused silica wall with a strong base to yield hydroxyl groups. After the condensation reaction the stationary phase is covalently bound to the inner capillary wall.

The Fused Silica Capillaries (manufacturer: Polymicro Technologies i.d. 100 µm, o.d. 360 µm or Optronis TSP 100 375, 100 µm ID, 363 µm OD) were cleaned and activated using the following procedure: 0,2M HCl for 30 min, distilled H_2O for 30 min, 1M NaOH for 90 min and distilled H_2O for 30 min.[42] An Isamtec Micropump was used to flush the capillaries.[43] To remove the liquid film inside the capillary which hinders the homogenate filling, the Si- capillary was put into an oven at 100°C for 20 min.

4.2 C8–functionalized Capillaries

The synthesis of the C_8-RP monolithic material included the following ratios of precursors and solvents. The choice of the ratio of precursors is important for the size of pores in the monolith.[50],[51]

The precursor mixture containing all compounds beside diethylamine was stirred at 60°C for 1 h in a glass vial. After cooling to room temperature, diethylamine was added. This mixture was immediately filled into the capillary according to the process described in 4.4.

Table 7. Ratio of educts for C_8-functionalized capillaries.

Compound	Mol equivalents (referring to TEOS)	Volume/Mass
TEOS (silica backbone)	1.00	900 µl
C_8-TEOS (reversed phase mode)	0.40	500 µl
0.5M HCl (catalyst for hydrolysis)	0.01	100 µl
H_2O (solvent)	1.25	100 µl
Polygosil particles (stabilizer)	15 wt%	0.45 g
MeOH (solvent)	22.2	1.80 ml
Diethylamine (catalyst for condensation)	0.25	25.0 µl

4.3 Aminofunctionalized Capillaries

The compounds (except 3-aminopropyltriethoxysilane (APTES)) were put into a glass vial and stirred for 2 min. After further homogenization in the ultrasonic bath for 1 min, APTES was added to the stirred reaction vessel. The mixture was then immediately charged into the capillary.

Table 8. Ratios of educts for amino-functionalized capillaries.

Compound	Mol equivalents (referring to TEOS)	Volume / Mass
TEOS	1	440 µl
H_2O	3.5	120 µl
MeOH	9	730 µl
Polygosil	15 wt%	0.2 g
APTES	0.5	230 µl

4.4 Filling

The precursor mixture was filled into the pretreated capillaries by putting the capillary and a syringe (or the outlet tube of the HPLC Pump) into the septum of a GC vial. The liquid was filled into the capillary by applying pressure with the help of the syringe (or with a Merck Hitachi L-6200 HPLC-Pump applying 1 bar).

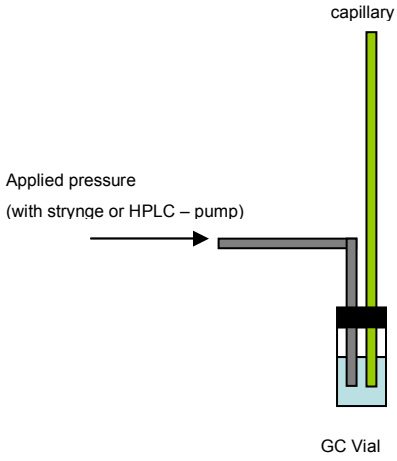


Figure 13. Principle of filling procedure.

25 cm of the capillary were filled with precursor mixture and 10 cm were left empty for creating a detection window with the use of a heated filament afterwards (see fig.14). After the precursor mixture was filled into the capillary, the capillary was sealed with silicone stoppers and left for polymerization over night at 40°C.

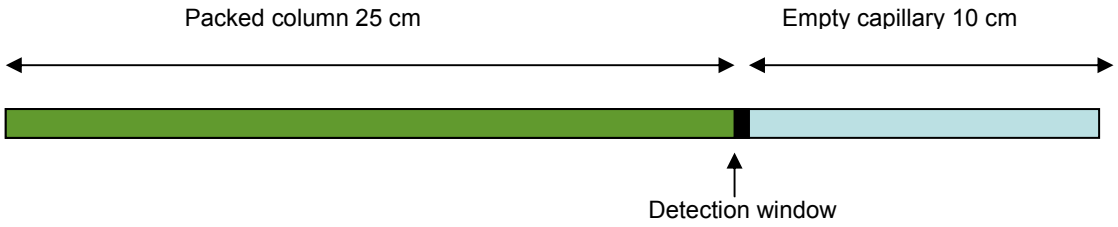


Figure 14. Schema of the filled capillary.

Finished Capillaries were tested for their pressure resistance at 50 bar (using a Merck Hitachi L-6200 HPLC-Pump, flushing liquid: MeOH). The majority of the C8 functionalized capillaries even withstood an applied pressure of 200 bar (maximum of the HPLC Pump).

The manufactured capillaries which were tested in Graz were implemented into a fully automated 3DCE system (Agilent Technologies, CA, USA) equipped with a diode array detector (Wavelenghts of 234, 240 and 450 nm were used for detection respectively). Capillaries tested at the TU Kaiserslautern were installed in an Agilent G1600A CE device with real time UV-Visible diode array detector (190-600nm).

4.5 Mobile Phase

A combination of MeOH and citrate buffer in certain ratios was used as stationary phase on the one hand, and on the other hand a mixture of acetonitril (ACN) and TRIS-buffer. The alkaline TRIS-buffer contained 25 mmol*L^{-1} tris-(hydroxymethyl)aminomethane (TRIS) and was prepared with bidistilled water. The acidic citrate buffer was prepared by mixing citric acid-1-hydrate (25 mmol*L^{-1}) with bidistilled water. The properties of the mobile phases are summarized in table 10. All solvents for the buffer preparation were obtained from VWR International (Darmstadt, Germany) and filtered with a syringe filter (0.2 µm pore size) and degassed by sonication before use.

Table 9. Mobile phases used and their properties at 25°C.

Mobile Phase	Ratio	Conductivity µS/cm	pH-value
TRIS/acetonitrile	1:4	356.0	8.00
citrate/methanol	1:4	112.9	4.77
citrate/methanol	1:9	65.8	5.06
citrate/methanol	1:19	34.4	5.27
citrate/methanol	1:29	28.8	5.37

5 Results

The following chromatograms and results can also be found in the deliverables 2.7 and 2.9 for the CAEC Continuous Annular Electro-Chromatography program (Grant Agreement NMP2-SL-2008-206707) of the Seventh Framework Program.

5.1 Characterization of the C_8 and Aminofunctionalized Stationary Phases

IR spectra

For characterizing the stationary phase the leftovers of the preparation of the stationary phases in the vial were examined via ATR using a Bruker VERTEX 70 ATR-FTIR, a DLaTGS detector and a MVP Pro Star, Diamond crystal ATR unit with a resolution of 4 cm^{-1}, 16 scans, 4000 – 600 cm^{-1}. The IR-spectra of the C_8 functionalized stationary phase can be seen in fig 15. It shows a Si – peak at 1000 - 1200 cm^{-1} and the peak of the methoxy and alkyl ligands at 2000-3000 cm^{-1}

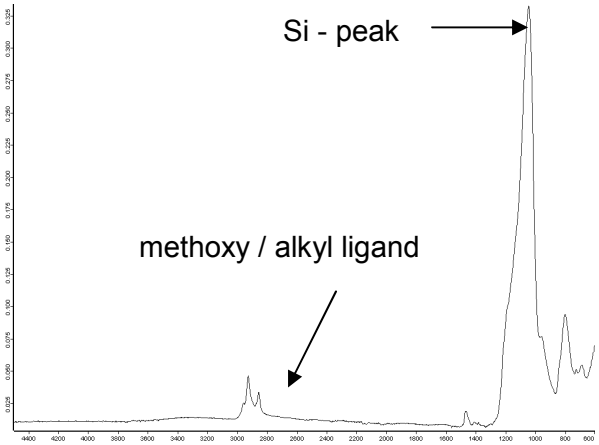


Figure 15. FTIR - spectrum of the C_8 –stationary phases.

The recorded spectra of the aminofunctionalized stationary phase can bee seen in fig 16. It again shows a Si – peak at 1000 - 1200 cm^{-1} and the peak of the methoxy and alkyl ligands at 2000-3000 cm^{-1}. Additionally the amino peaks at 1400 -1600 cm^{-1} and 3100 – 3300 cm^{-1} indicate a successful aminofunctionalization.

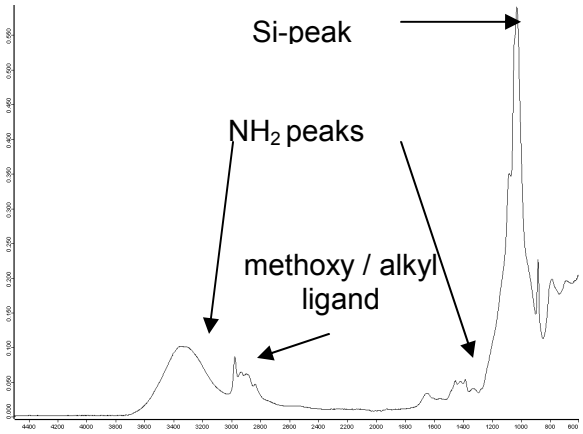


Figure 16. FTIR - spectrum of the aminofunctionalized stationary phases.

Visual Examination

As for visual examination of the integrity of the stationary phase an optical microscope Leica DM4000 M equipped with a Leica DFC290 CMOS camera was used. The picture seen in fig 17 was processed with Leica Application Suite, Version 2.7.1.

SEM pictures were taken with a FEI Quanta 600 FEG-ESEM system and a NORAN Vantage system with Si(Li)-detector (10 mm2, PlexUs 0.65um window, energy resolution 132eV)

Figure 17. 50 times magnified picture of an empty (top) and filled (bottom) capillary.

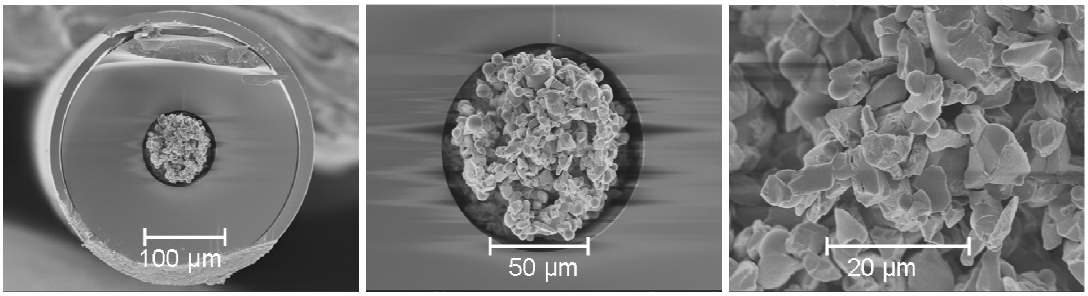


Figure 18. SEM (Scanning Electron Microscopy) pictures of a CEC capillary filled with C8 functionalized stationary phase.

BET / Porosity

BET analysis of the surface area of the manufactured C_8 monolith was carried out with an ASAP 2000 micromeritics device.

The hysteresis between adsorption and desorption curve construes the existence of mesopores. In general the explanation for the hysteresis loop in type IV and V (fig. 19) is the capillary condensation of the adsorbate in the mesopores of the solid. As it can be seen in figure 19, the measured isotherm complies with type IV. According to the results in table 10 and the definition issued by the IUPAC[53],

- micropores: < 2nm
- mesopores: 2 – 50 nm
- macropores: > 50nm

the manufactured monolith is mesoporouse.

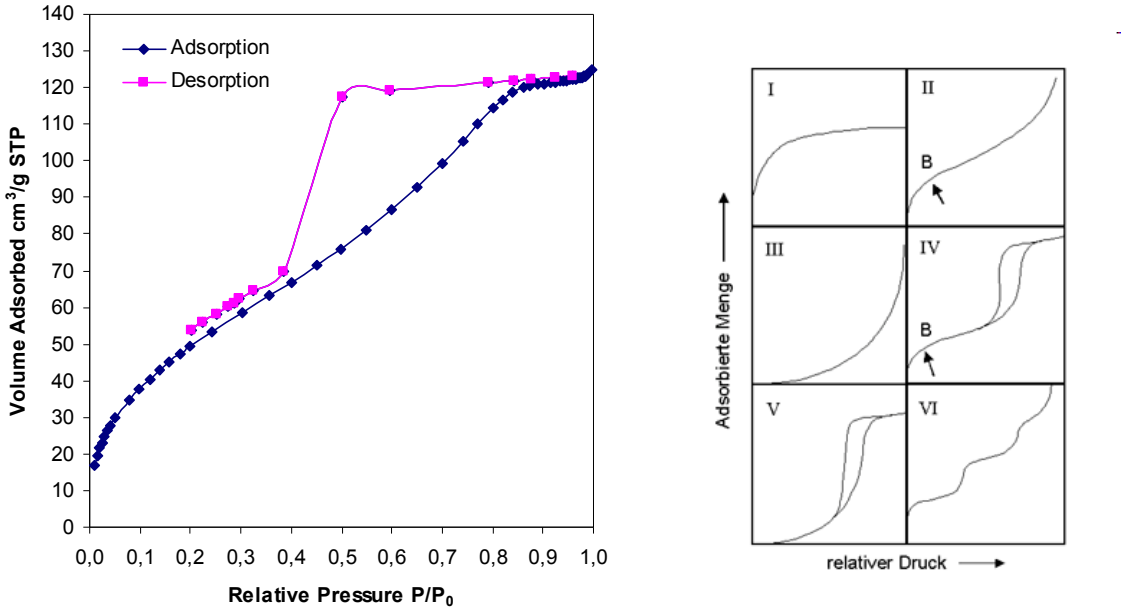


Figure 19. left: Isotherm plot of the BET analysis of the manufactured C_8 stationary phase. Right: types of sorptionisotherms according to IUPAC[52].

Table 10. Results of the BET Analysis. Values are average values of 3 runs.

BET Surface Area [m^2*g^{-1}]	Pore Volume [cm^3*g^{-1}]	Pore Diameter [nm]
126.96 ± 0.54	0.1651 ± 0.0008	5.2029 ± 0.0025

5.2 Concentration Recovery Experiment

One problem in CEC is the reproducibility of measurements. Due to the fact that the injection procedure is based on electric forces, it is difficult to always attain the same volume of sample to the capillary.[5] One way to better influence the amount of the sample brought into the capillary is to use pressure additionally to the electrical application, while the separation itself runs on the thrive of electrical forces only. To see if the CE - chromatograph and the injection run on a steady level, a concentration recovery experiment was carried out. For this purpose a set concentration of anthracene was measured with a C_8-functionalized capillary, after a preconditioning phase of 2 minutes with 12 bar from inlet to outlet, the measurements were carried out at 15kV applied voltage, pressure was 12 bar at inlet and outlet, injection at 20kV for 5 seconds, mobile phase was TRIS/ACN (tris(hydroxymethyl)aminomethane / acetonitrile) 1:4. The results of the experiment can be seen in table 11 and figure 20.

Table 11. Peak areas for certain concentrations of anthracene, detected at λ= 240,16 nm.

		Area				
Sample	**Concentration [mg/ml]**	**Run 1**	**Run 2**	**Run 3**	**Mean**	**Deviation**
1	0.4536	7622.2	9954.2	6580.0	8052.1	1727.7
2	0.3402	5000.3	6863.2	7458.0	6440.5	1282.2
3	0.2268	3008.3	2866.3	4338.2	3404.2	811.92
4	0.1134	1787.8	2352.1	3019.9	2386.6	616.77
5	0.0454	718.40	1137.1	807.6	887.70	220.54

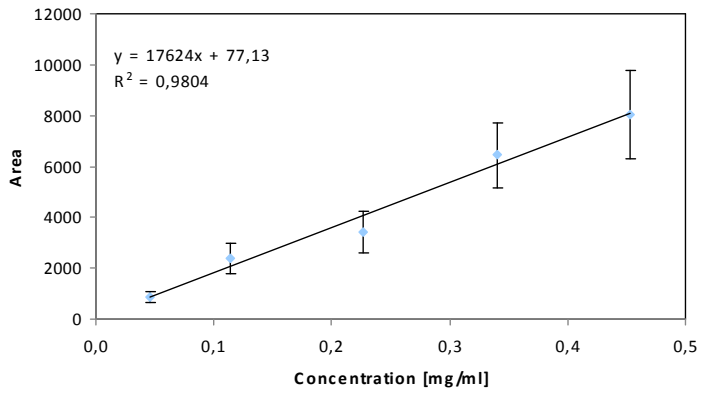


Figure 20. Results of the concentration recovery experiment.

As fig 20 shows it is possible to obtain a reproducible result (coefficient of determination R^2 = 0.98), despite the bad reputation of CEC regarding injection precision. Higher deviation of the samples 1 and 2 might be correlated to detector sensitivity regarding higher concentrations.

5.3 Separation of Thiourea, Naphthalene and Anthracene

For testing the manufactured C_8 – capillaries in terms of efficiency, reproducibility and selectivity, a test mixture of naphtalene and anthracene was used. Thiourea was added as tracer. A representative chromatogram (using TRIS/ACN = 1:4 as mobile phase) can be seen in fig 21. Further the same sample mixture was tested under different mobile phase conditions (See table 12). Also the applied voltage on the column during the separation run was altered. Results regarding efficiency, reproducibility and dependence of retention time on mobile phases and applied voltage are shown in table 12 and 13.

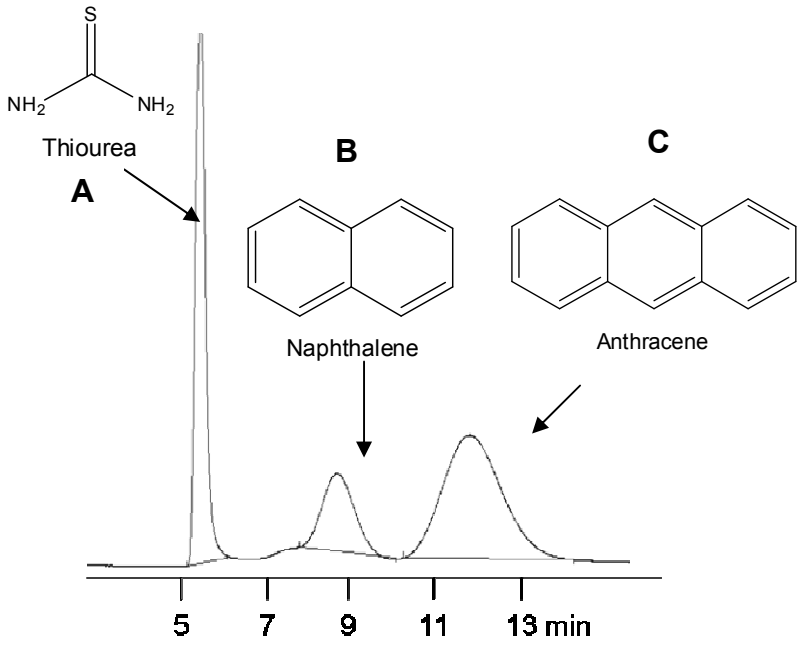


Figure 21. Chromatogram of A: thiourea, B: naphthalene and C: anthracene, mobile phase TRIS/ACN = 1:4

Table 12. Influence of mobile phase and mobile phase composition on retention times of test molecules. Voltage is 20kV. Values are average values of at least 3 runs. Compounds: A: thiourea, B: naphthalene, C: anthracene.

	A	**B**	**C**			**B**	**C**
	t_R [min]	t_R [min]	t_R [min]	α	Rs	N/m	N/m
TRIS/ACN 1:4	5.81	8.78	10.97	1.25	2.90	~13400	~9400
Cit/MeOH 1:4	68.43	177.08	215.8	1.19	2.81	~16800	~16400
Cit/MeOH 1:9	36.72	59.012	68.28	1.16	2.44	~19500	~17200
Cit/MeOH 1:19	23.56	32.15	36.61	1.14	2.29	~16400	~17300
Cit/MeOH 1:29	17.86	24.71	29.74	1.20	3.12	~20100	~17200

As it can be seen in table 12 the TRIS/ACN mobile phase yielded the best results regarding fast retention times with the thiourea / naphthalene / anthracene mixture. The higher the content of citrate buffer, the lower the pH value of the mobile phase and thus the longer the retention time.

Table 13. Influence of applied voltage on retention times of test molecules. Mobile phase is Cit/MeOH = 1:29. Compounds: A: thiourea, B: naphthalene, C: anthracene.

	A	**B**	**C**			**B**	**C**
	t_R [min]	t_R [min]	t_R [min]	α	Rs	N/m	N/m
10 kV	42.9	56.3	63.7	1.55	4.35	~24300	~31000
20 kV	17.9	24.6	29.7	1.20	3.12	~20100	~17200
30 kV	11.3	15.0	17.0	1.54	2.68	~21200	~6200

Apparently the applied voltage enhances the EOF and thus lowers the retention times of the test molecules. What could also be observed was a decrease of the theoretical plates, but this trend was only significantly present with the anthracene peaks.

For determining the reproducibility of retention times a C_8 - functionalized capillary was tested with the thiourea / naphthalene / anthracene testmixture for six runs. Results can be seen in fig 22, the achieved theoretical plates of the same experiment can be seen in fig 23.

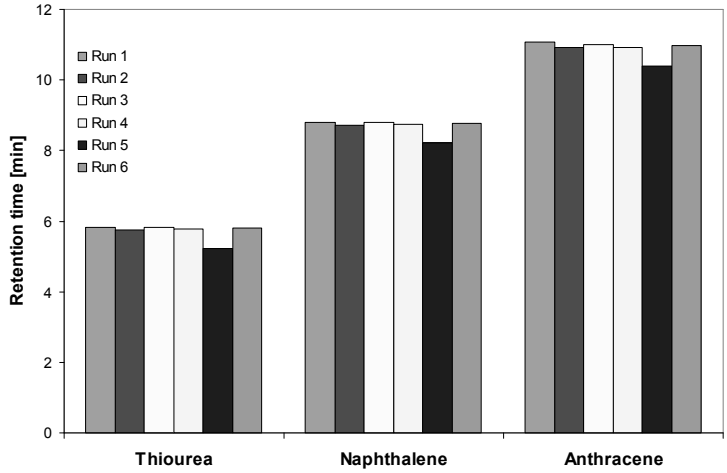


Figure 22. Reproducibility of C_8–functionalized capillary number 2. Mobile phase is TRIS/ACN = 1:4, voltage is 20 kV.[54]

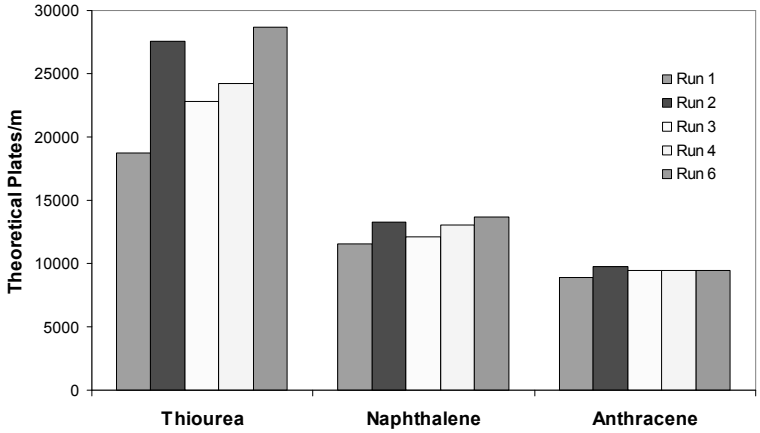


Figure 23. Retention time of C_8 – functionalized capillary number 2. Mobile phase is TRIS/ACN = 1:4, voltage is 20 kV.[54]

Figure 22 and 23 as well as the concentration recovery experiment (point 4.4) proof that CEC can be operated at a steady level without much deviation. Now as the reproducibility of the CEC is demonstrated, the packing of the columns was investigated.

Table 14. Comparison between capillaries regarding retention times. Mobile phase is TRIS/ACN = 1:4, voltage is 20kV. Values are average values of three runs.

	t_R naphthalene [min]	t_R anthracene [min]
Capillary 1	8.66 ± 0.09	11.09 ± 0.11
Capillary 2	8.67 ± 0.23	10.88 ± 0.24
Capillary 3	9.00 ± 0.56	11.60 ± 0.71

Table 15. Comparison between capillaries regarding theoretical plates per meter. Mobile phase is TRIS/ACN = 1:4, voltage is 20kV.

	Theoretical plates/m naphthalene	Theoretical plates/m anthracene
Capillary 1	16740 ± 528	13812 ± 561
Capillary 2	12725 ± 857	9408 ± 326
Capillary 3	13868 ± 1131	12081 ± 1116

Tables 14 and 15 show that the C_8 - functionalized silica monolith via the sol gel process and the filling procedure into the capillary yield an efficient and robust stationary phase. Further testing focused on the selective separation of common basic chemicals.

Setting the focus on the selectivity of the manufactured C_8-columns, three different multi-component test mixtures were used. Measurements (with one exception) took place at the University of Kasierslautern.

1. Polycyclic aromatic hydrocarbons (PAHs)*
2. Alkylbenzenes
3. Phenols

*measured in Graz, KFU, working group of Hrn. Ao. Univ.-Prof. Dr. Martin Schmid of the Institut für Pharmazeutische Wissenschaften.

5.4 Polycyclic Aromatic Hydrocarbons (PAH) Test Mixture

PAHs are suspected of inducing cancer in humans.[55] By the epoxidation of the PAHs in the human metabolism, these compounds interfere with the DNA and cause defects in the cell cycle. Oversaturated samples of thiourea, naphthalene, acenaphthylene, phenantrene, anthracene, fluoranthene and pyrene were combined in one vial (filtered through a 0.2 µm syringe filter to remove solids in the liquid) and the CEC experiment was carried out (after a preconditioning phase of 2 minutes with 12 bar from inlet to outlet) at 15kV, pressure was 12 bar at inlet and outlet, injection at 20kV for 5 seconds, mobile phase was TRIS/ACN 1:4. Results can be seen in fig 24 and table 16.

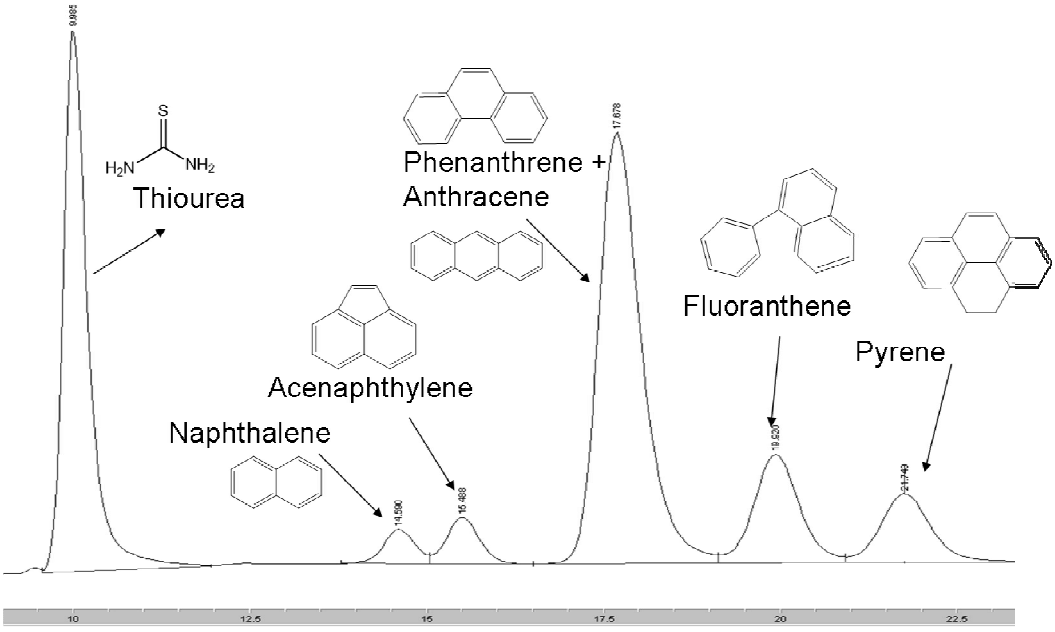


Figure 24. Chromatogram of the PAH test mixture.[56]

Table 16. Retention times and theoretical plates per meter of C_8-functionalized monolithic column. Values are average values of three runs. A: thiourea, B: naphthalene, C: acenaphthylene, D: phenantrene, E: anthracene, F: fluoranthene and G: pyrene. Values are average values of three runs.

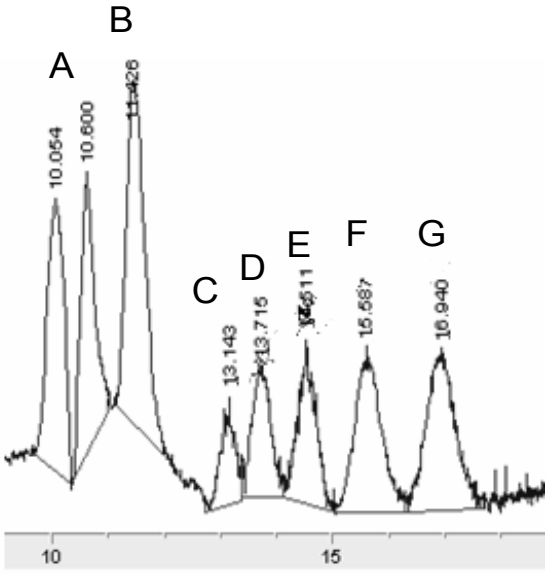
	A	**B**	**C**	**D + E**	**F**	**G**
t_R [min]	9.76 ± 0.23	14.23 ± 0.31	15.07 ± 0.36	17.23 ± 0.40	19.45 ± 0.41	21.25 ± 0.43
N/m	14200 ± 5900	18000 ± 3500	14400 ± 1000	7600 ± 580	8300 ± 1000	8914 ± 700
R_S	-	2.94 ± 0.29	0.73 ± 0.09	1.17 ± 0.07	1.07 ± 0.06	0.94 ± 0.15

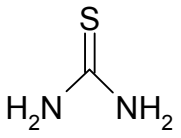
The separation of the PAH testmixture was successful and base line separated, except the overlap of the phenanthrene peak with the anthracene peak, reason for this might be the minimal difference in polarity due to the structure of the molecules.

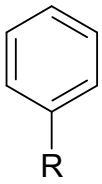
5.5 Alkylbenzene Test Mixture

Alkylbenzenes and phenols are common contaminants in the chemical and pharmaceutical production. Therefore, the manufactured stationary phases were also tested for the separation of these two types of chemicals.

83mg/L samples of thiourea, benzene, methylbenzene, ethylbenzene, propylbenzene, butylbenzene and pentylbenzene were combined in one vial and the CEC experiment was carried out at 20kV, injection at 10kV for 3 seconds, mobile phase was TRIS/ACN 1:9. Results can be seen in fig 25 and table 17.







A thiourea **B** benzene: R = H

C methylbenzene: R=CH_3,

D ethylbenzene: R=CH_2CH_3,

E propylbenzene R=$(CH_2)_2CH_3$,

F butylbenzene R=$(CH_2)_3CH_3$,

G pentylbenzene R=$(CH_2)_4CH_3$

Figure 25. Chromatogram alkylbenzene test mixture (TU Kaiserslautern).

Table 17. Retention times, theoretical plate number and resolution of the alkylbenzene test mixture. Data are average values of 3 runs.

	A	**B**	**C**	**D**	**E**	**F**	**G**
t_R [min]	10.60 ± 0.01	11.41 ± 0.02	13.11 ± 0.04	13.69 ± 0.03	14.47 ± 0.05	15.56 ± 0.04	16.87 ± 0.10
N/m	25574 ± 7187	17200 ± 1245	37910 ± 2750	22852 ± 2749	27250 ± 9376	19192 ± 6370	17780 ± 3536
R_S	-	1.33 ± 0.13	2.67 ± 0.56	0.89 ± 0.18	1.10 ± 0.15	1.34 ± 0.21	1.37 ± 0.23

The separation of the alkylbenzene testmixture was successful, although not base line separated as it was the case with the PAH system.

5.6 Phenol Test Mixture

100mg/L samples of phenol, 3,5-dimethoxyphenol, 2,5-dimethyl-4-(morpholinomethyl)-phenol mono-(3-chlorophenyl)-phenylmethylphtalat, 2-nitrophenol, and 4,6-dichloro-2-(5-isoxalyl)-phenol) were combined in one vial and the CEC experiment was carried out at 20kV, injection at 10kV for 3 seconds, mobile phase was Cit/ACN = 1:9. Results can be seen in fig 26 and table 18.

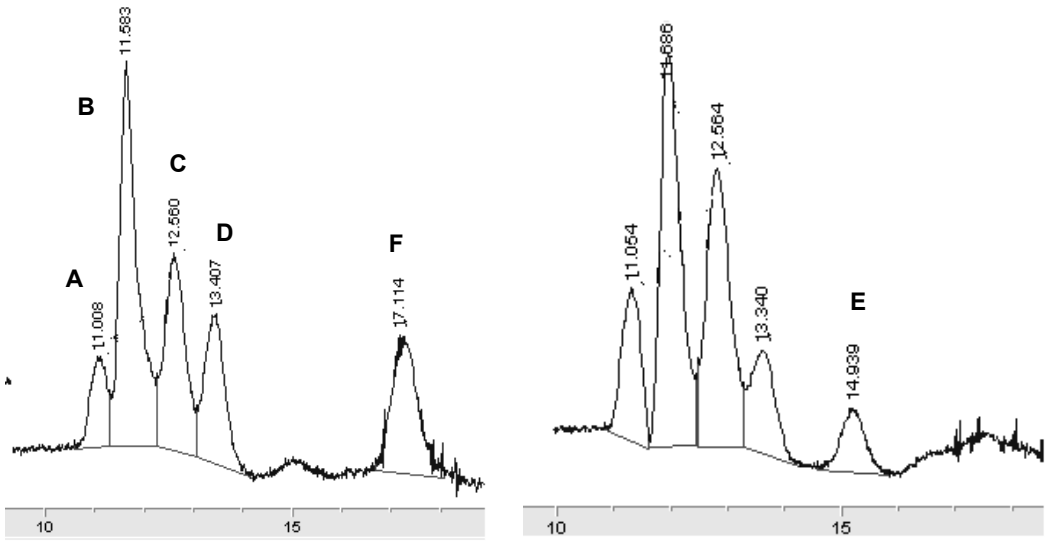
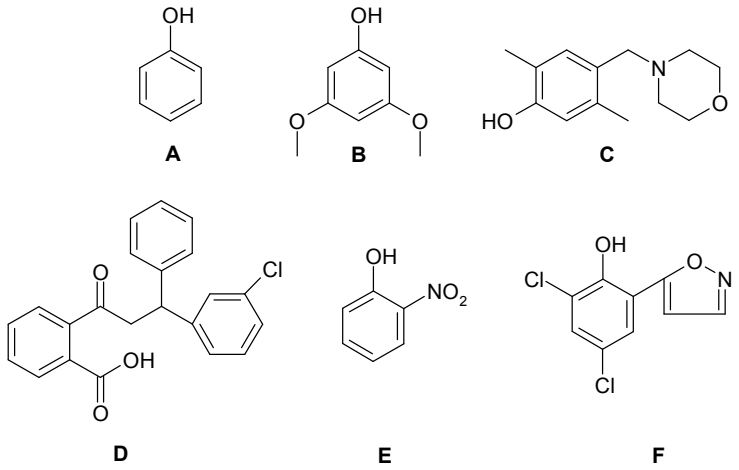


Figure 26. Chromatograms of the phenol test mixture at (left) 212 nm and (right) 235 nm (TU Kaiserslautern).



A phenol **B** 3,5-dimethoxyphenol **C** 2,5-dimethyl-4-(morpholinomethyl)-phenol

D mono-(3-chlorophenyl)-phenylmethylphtalat **E** 2-nitrophenol **F** 4,6-dichloro-2-(5-isoxalyl)-phenol

Table 18. Retention times, theoretical plate number and resolution of the phenol testmixture. Data represent average values of 2 runs. Compounds: A: phenol, B: 3,5-dimethoxyphenol, C: 2,5-dimethyl-4-(morpholinomethyl)-phenol D: mono-(3-chlorophenyl)-phenylmethylphtalat, E: 2-nitrophenol, F: 4,6-dichloro-2-(5-isoxalyl)-phenol.

	A	**B**	**C**	**D**	**E**	**F**
t_R [min]	10.82 ± 0.33	11.50 ± 0.26	12.32 ± 0.34	13.14 ± 0.29	14.72 ± 0.31	16.93 ± 0.27
N/m	14466 ± 2695	16546 ± 4262	15176 ± 1035	15222 ± 2554	31834 ± 11967	22128 ± 2025
R_S	-	0.94 ± 0.01	1.09 ± 0.16	0.98 ± 0.11	2.08 ± 0.29	4.58 ± 0.49

Again the separation of the phenol test mixture was successful, although not base line separated as it was the case with the PAH system.

5.7 PAH Testsystem under optimized Conditions

In general it can be seen that each and every separation task requires its own conditions regarding applied voltage and mobile phase (composition). To illustrate this, the conditions for the PAH separations (point 5.4) were altered by the TU Kaiserslautern. The results can be seen in fig 27 to 29.

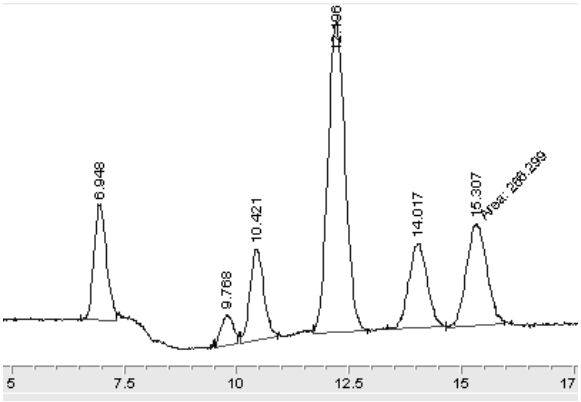


Figure 27. Electrochromatogram of the PAH test system. Sample concentration: 83mg/L; injection: 10kV, 6 s; voltage: 17 kV; electrolyte: H_2O:MeOH = 1:9. (TU Kaiserslautern).

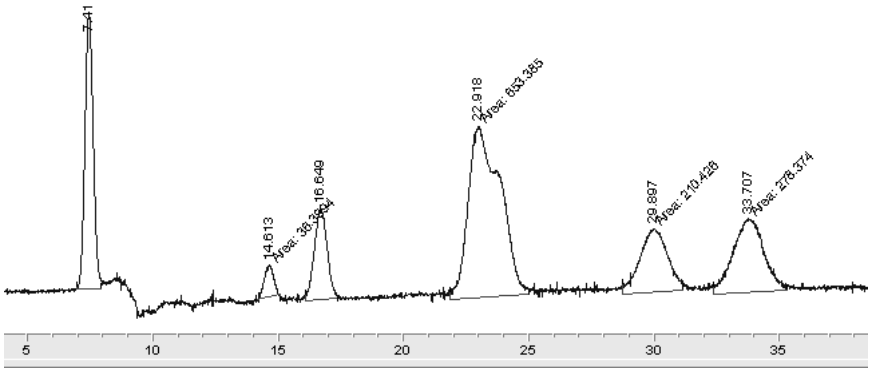


Figure 28. Electrochromatogram of the PAH test system. Sample concentration: 83mg/L; injection: 10kV, 6 s; voltage: 17 kV; electrolyte: H_2O:MeOH = 1:4. (TU Kaiserslautern).

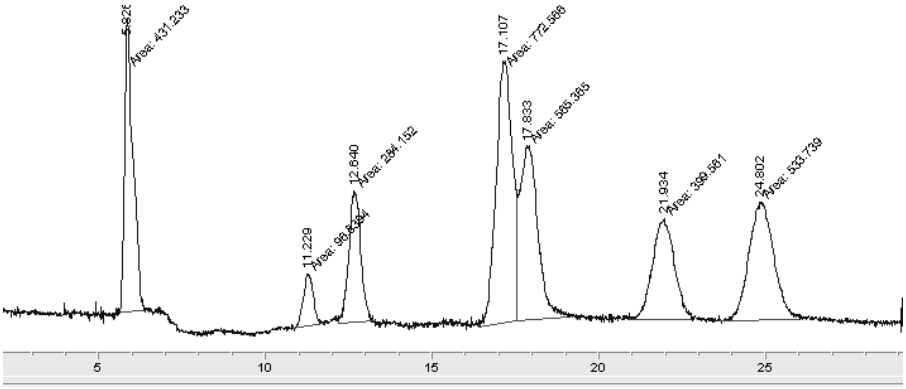


Figure 29. Electrochromatogram of the PAH test system. Sample concentration: 83mg/L; injection: 10kV, 6 s; voltage: 20 kV; electrolyte: ACN:MeOH = 1:3 + 20% H_2O. (TU Kaiserslautern).

Table 19. Data of the chromatograms from fig.26 – 28. A: thiourea, B: naphthalene, C: acenaphthylene, D: phenantrene, E: anthracene, F: fluoranthene and G: pyrene.

	Mobile phase	**A**	**B**	**C**	**D + E**	**F**	**G**
t_R [min]	H_2O:MeOH = 1:9	6.948	9.768	10.421	12.196	14.017	15.307
	H_2O:MeOH = 1:4	7.410	14.613	16.649	22.918	29.897	33.707
	ACN:MeOH = 1:3 + 20% H_2O	5.826	11.229	12.64	**17.107 +17.833**	21.934	24.802
N/m	H_2O:MeOH = 1:9	12064	19416	22552	18988	21500	21624
	H_2O:MeOH = 1:4	9744	25996	19356	4824	13456	13348
	ACN:MeOH = 1:3 + 20% H_2O	10316	25660	23684	**16512 + 17388**	22176	21660
R_S	H_2O:MeOH = 1:9	-	5.28	1.17	2.81	2.47	1.61
	H_2O:MeOH = 1:4	-	10.85	2.42	3.48	2.96	1.73
	ACN:MeOH = 1:3 + 20% H_2O	-	10.58	2.31	**5.18 + 0.68**	3.62	2.27

Adding water to the electrolyte improved the separation of the single compounds, most important, a better separation of phenanthrene and anthracene could be achieved, although resolution values did not rise above 1.

The electrolyte combination of ACN:MeOH = 1:3 + 20% H_2O proved to be the most efficient one regarding both selectivity (7 discrete peaks) and efficiency (highest plate number of all runs) for the PAH testsystem.

5.8 Calcitonin

Calcitonin is a 32-amino acid linear polypeptide hormone that participates in the calcium (Ca2+) and phosphorus metabolism. Therapeutic uses are Hypercalcaemia, (postmenopausal) osteoporosis, Paget's disease (infants), bone metastases and Phantom limb pain.[57] A sample of calcitonin was provided to the TU Kaiserslautern by NOV.

The injection of the calcitonin sample into the negatively charged C_8 - functionalized column proved to be not possible, not by pressure, nor by voltage. Therefore, the aminofunctionalized monolith was tested.

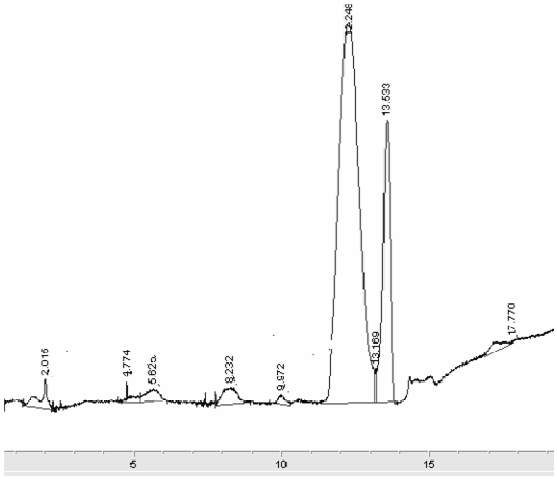


Figure 30. Chromatogram of calcitonin using an aminofunctionalized monolithic stationary phase. Sample concentration: 1g/L; injection: 12bar, 0.7min; voltage: 20 kV; electrolyte: ACN:MeOH = 3:1 with 5% of 7.5mM TRIS pH = 8. (TU Kaiserslautern).

Unfortunately, at the time of writing this thesis, there was no reference chromatogram available and there was no info about how many compounds should be separated.

5.9 Comparison with Commercial Stationary Phase

In order to compare the separation efficiency of the silica based monolith to a commercial available CEC capillary, the PAH and the phenol mixture were separated under the same conditions regarding mobile phases and applied voltage using an Agilent CEC Cap HypC8 3µm, 100µm / 25cm 2 / PK capillary.

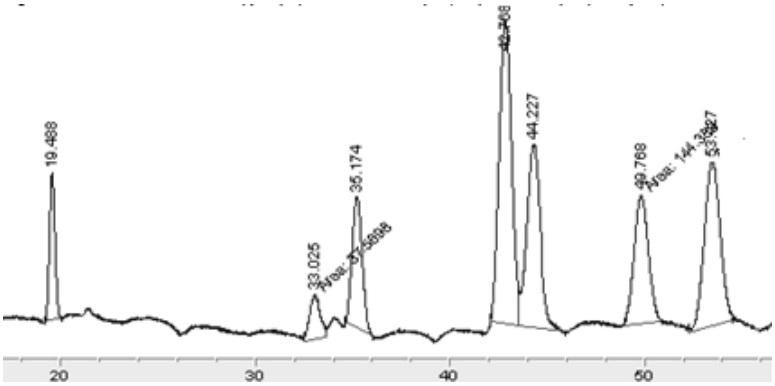


Figure 31. Chromatogram of the PAH test system using a C_8-Agilent capillary. Sample concentration: 83mg/L; injection: 10kV, 6 s; voltage: 17 kV; electrolyte: H2O:MeOH = 1:4.

In table 20 the retention times, theoretical plate numbers and resolutions of the separation of the PAH mixture using an Agilent C8 capillary are summarized in **red** letters, values of the manufactured C8 monolith are printed in **blue** letters. Compounds were A: thiourea, B:naphthalene, C: acenaphthylene, D: phenantrene, E: anthracene, F: fluoranthene and G: pyrene.

Table 20. Separation of the PAH test mixture, Sample concentration: 83mg/L; injection: 10kV, 6 s; voltage: 17 kV; electrolyte: H_2O:MeOH = 1:4. Data represents average values of 5 runs for the Agilent column.

	A	B	C	D	E	F	G
t_R (min)	19.43 ±0.82	32.87 ±1.25	35.02 ±1.34	42.60 ±1.60	44.03 ±1.63	49.55 ±1.76	53.17 ±1.87
t_R (min)	7.410	14.613	16.649	22.918		29.897	33.707
N (m^{-1})	80510 ± 4215	80005 ± 13961	81982 ± 4209	82396 ± 6861	76555 ± 2636	84973 ± 5211	83030 ± 4638
N (m^{-1})	9744	25996	19356	4824		13456	13348
R_S	-	18.12 ± 1.02	2.24 ± 0.04	6.99 ± 0.20	1.16 ± 0.01	4.19 ± 0.10	2.55 ± 0.08
R_S	-	10.85	2.42	3.48		2.96	1.73

What can be affiliated from the data printed in table 20 is that the Agilent column achieves a higher resolution and a higher plate number but the compounds were not base – line separated. Also phenanthrene and anthracene showed two distinct peaks in contrast to the manufactured C_8 monolithic column. However one drawback in a continuous set- up with the annular chromatograph could be the longer (ca. 20 min by the end of separation) retention times under the experimental conditions.

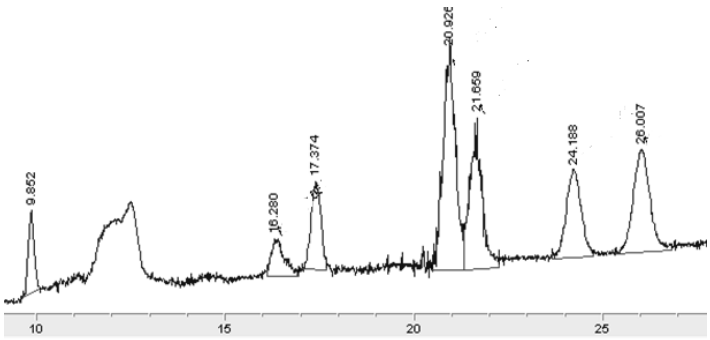


Figure 32. Chromatogram of the PAH test system using a C_8-Agilent capillary. Sample concentration: 83mg/L; injection: 10kV, 6 s; voltage: 20 kV; electrolyte: ACN:MeOH = 1:3 + 20% H_2O.

In table 21 there are retention times, theoretical plate numbers and resolutions of the separation of the PAH mixture using an Agilent C8 capillary in **red** letters, values of the manufactured C8 monolith are printed in **blue** letters.

Table 21. Separation of the PAH test mixture Sample concentration: 83mg/L; injection: 10kV, 6 s; voltage: 20 kV; electrolyte: ACN:MeOH = 1:3 + 20% H_2O. Data represents average values of 4 runs for the Agilent column.

	A	B	C	D	E	F	G
t_R (min)	10.13 ± 0.27	16.71 ± 0.42	17.79 ± 0.42	21.39 ± 0.48	22.11 ± 0.48	24.70 ± 0.52	26.49 ± 0.51
t_R (min)	5.826	11.229	12.64	17.107	17.833	21.934	24.802
N (m^{-1})	87024 ± 12135	50003 ± 9820	80839 ± 5720	77623 ± 6510	75285 ± 2215	75046 ± 5597	76215 ± 6288
N (m^{-1})	10316	25660	23684	16512	17388	22176	21660
R_S	-	14.98 ± 1.26	1.96 ± 0.13	6.44 ± 0.21	1.14 ± 0.06	3.79 ± 0.08	2.39 ± 0.04
R_S	-	10.58	2.31	5.18	0.68	3.62	2.27

The data presented in table 21 shows that the Agilent column has higher resolution and a higher plate number but the compounds were not base – line separated.

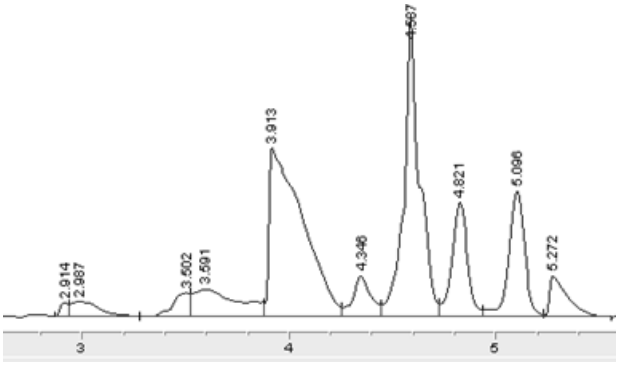


Figure 33. Chromatogram with the phenol test system using a C_8-Agilent capillary. Sample concentration: 100mg/L; injection: 10kV, 3 s; voltage: 20 kV; electrolyte: Cit:ACN = 1:9.

In table 22 there are retention times, theoretical plate numbers and resolutions of the separation of the phenol mixture using an Agilent C8 capillary in **red** letters, values of the manufactured C8 monolith are printed in **blue** letters. Compounds were A: Phenol, B: 3,5-dimethoxyphenol, C: 2,5-dimethyl-4-(morpholinomethyl)-phenol, D: mono-(3-chlorophenyl)-phenylmethylphtalat, E: 2-nitrophenol, F: 4,6-dichloro-2-(5- isoxaolyl)-phenol.

Table 22. Separation of the phenol test mixture. Sample concentration: 100mg/L; injection: 10kV, 3 s; voltage: 20 kV; electrolyte: Cit:ACN = 1:9. Data represents average values of 5 runs for the Agilent column and 3 for the manufactured C_8 monolithic column.

	A	B	C	D	E	F
t_R (min)	3.95 ± 0.04	4.38 ± 0.04	4.61 ± 0.03	4.85 ± 0.03	5.13 ± 0.03	5.31 ± 0.05
t_R (min)	10.82 ± 0.33	11.50 ± 0.26	12.32 ± 0.34	13.14 ± 0.29	14.72 ± 0.31	16.93 ± 0.27
N (m^{-1})	10780 ± 1444	32849 ± 23262	105545 ± 59580	85640 ± 1938	96241 ± 447	87620 ± 15289
N (m^{-1})	14466 ± 2695	16546 ± 4262	15176 ± 1035	15222 ± 2554	31834 ± 11967	22128 ± 2025
R_S	1.10 ± 0.15	1.71 ± 0.28	1.42 ± 0.60	1.95 ± 0.20	2.06 ± 0.04	1.28 ± 0.10
R_S	-	0.94 ± 0.01	1.09 ± 0.16	0.98 ± 0.11	2.08 ± 0.29	4.58 ± 0.49

The data presented in table 22 shows that the Agilent column has higher resolution and a higher plate number as well as shorter retention times.

6 Summary and Outlook

Two different monolithic stationary phases for CEC were produced. One with a C_8- the other with amino - functionalization. The stationary phase was characterized by light microscopy and SEM. Additionally, an IR spectra and a BET analysis was done to characterize the manufactured monoliths.

The performance of the manufactured capillary monolithic column can be summarized as follows:

- PAH separation: Using ACN:MeOH = 1:3 + 20% H_2O as mobile phase all compounds of the test mixture were base line separated. Theoretical plates were up to 25600 per meter, however, the separation coefficient of phenantrene and anthracene was only 0.68.
- Alkylbenzene mixture separation: By using TRIS:ACN = 1:9 the compounds were separated although not base line separated. Theoretical plate number ranged up to 38000 per meter and resolution were >1 so that all peaks emerged discrete on the chromatogram.
- Phenol mixture separation: The separation with Cit/ACN = 1:9 as mobile phase showed theoretical plates up to 32000 per meter but the peaks were not base line separated and the obtained resolutions were below 1.
- Calcitonin separation: Using ACN:MeOH = 3:1 with 5% of 7.5mM TRIS pH = 8 as mobile phase, the compounds of the calcitonin mixture were separated. Unfortunately at the time of writing this thesis there was no data available what compounds exactly were separated.

All in all the new stationary phase proved to be convenient for the separation of different compounds.

The next step in this project would be the implementation of the developed stationary phases into the annular prototype (seen in fig 1) and the continuous separation of different compound mixtures.

7 List of Chemicals and Analytical Devices used

tetraethoxysilane	(TEOS, 95% Fluka)
triethoxy(octyl)silane	(C8-TEOS, 97.5%, Aldrich)
polygosil particles	60-5 5µm (Lactan)
APTES	(99% Aldrich)
diethylamine	(99.5%, Fluka)
water	(ultrapure, 0.06 µs/cm)
HCl	(32% p.a., Roth)
NaOH	(99%, p.a.)
MeOH	(99%, Roth)
naphthalene	(98%, Fluka)
anthracene	(96%, Fluka)
thiourea	(99%, Aldrich)

Fused Silica Capillaries: Polymicro Technologies i.d. 100 µm, o.d. 360 µm
Optronis TSP, i.d. 100 µm , o.d. 363 µm

PAHs (Polycyclic aromatic hydrocarbons): Naphthalene (98%, Fluka), acenaphthylene (75%, Aldrich), phenanthrene (98%, Aldrich), anthracene (96%, Fluka), pyrene (98%, Aldrich), fluoranthene (98%, Aldrich).

Alkylbenzene mixture: Benzene (99.9%, Sigma-Aldrich), toluene (99.9%, Sigma-Aldrich), ethylbenzene (>99%, Fluka), propylbenzene (98%, Aldrich), butylbenzene (99%, Aldrich), pentylbenzene (99%, Aldrich).

Phenol mixture: Phenol (99.5%, Aldrich), 3-(o-Methoxyphenoxy)-1,2-propanediol (98%, Aldrich), 3,5-dimethoxyphenol (99%, Aldrich), 2-nitrophenol (99%, Fluka), 2,5-dimethyl-4-(morpholinomethyl)-phenol (technical grade, Aldrich), 2,4-dichloro-6-(5- isoxazolyl)-phenol (97%, Aldrich).

An **Ismatec Micropump** was used to flush the capillaries.

For IR measuring a **Bruker VERTEX 70 ATR-FTIR, a DLaTGS detector** and a **MVP Pro Star, Diamond crystal ATR unit** was used.

A **Merck Hitachi L-6200 HPLC-Pump** was used to fill the capillaries.

An optical microscope **Leica DM4000 M** equipped with a **Leica DFC290 CMOS camera** was used. Pictures were processed with **Leica Application Suite, Version 2.7.1.**

CEC device used at the KF Graz was a **fully automated 3DCE system (Agilent Technologies, CA, USA)** equipped with a diode array detector.
CEC device used at the TU Kaiserslautern was an **Agilent G1600A CE device** with real time UV-Visible diode array detector (190-600nm).

BET measuring was carried out with an **ASAP 2000 micromeritics** device.

SEM pictures were taken with a **FEI Quanta 600 FEG-ESEM** system and a **NORAN** Vantage system with Si(Li)-detector (10 mm2, PlexUs 0.65um window, energy resolution 132eV)
The commercial CEC capillary used was an **Agilent CEC Cap HypC8 3µm, 100µm / 25cm 2 / PK** capillary

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