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**NANOPARTICLES AS A TOOL
IN
CAPILLARY ELECTROCHROMATOGRAPHY**

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Abstract

Two different types of molecularly imprinted nanoparticles against (R)-propranolol were used to separate the enantiomers of propranolol in capillary electrochromatography mode, methacrylic acid based nanoparticles and core-shell molecularly imprinted polymer nanoparticles. Partial filling technique was used to avoid interference of molecularly imprinted polymer nanoparticles in UV detection.

With methacrylic acid based nanoparticles it was not possible to obtain enantiomer separation. Strong unspecific interactions between the molecular imprinted polymer nanoparticles and propranolol disturbed enantiomer separation. Since large content of acetonitrile had to be used in order to obtain stable suspensions of molecularly imprinted polymer nanoparticles, the electrostatic interactions were favored which contributed to the unspecific interactions occurring.

Core-shell molecularly imprinted polymer nanoparticles present suspension stability at low content of acetonitrile due to the poly(acrylamide) shell that makes them more hydrophilic. Enantiomer separation of propranolol was achieved with 40% of acetonitrile. Reproducibility was problematic due to the unspecific interactions occurring. With time several factors can occur contributing to the decreased reproducibility of results such as, interactions between the molecularly imprinted polymer nanoparticles and the capillary wall or evaporation of the organic solvent due to the design of vials used in capillary electrochromatography system.

The core-shell molecularly imprinted polymer nanoparticles are more suitable for propranolol enantiomer separation in comparison to methacrylic acid based nanoparticles. More stable suspensions give a greater range of conditions that can be tested.

Silanized gadolinium oxide nanoparticles were tested as pseudostationary phase in capillary electrochromatography for protein separation. The lack of interference with UV detection and the large surface area of these nanoparticles make them a promising tool in capillary electrochromatography for protein separation. These nanoparticles interact with the proteins that are analyzed. Increased injection times of the nanoparticles give retained

peaks of human growth hormone showing that strong interactions between the protein and nanoparticles are occurring.

Lysozyme that was not recovered using conventional capillary electrophoresis could be detected when nanoparticles were used as pseudostationary phase. The nanoparticles can act as a coating in the capillary wall or due to their large surface area they can prevent adsorption of the lysozyme to the capillary.

Resumo

Dois tipos diferentes de nanopartículas impressas molecularmente, selectivas de (R) – propranolol, foram utilizadas para a separação dos enantiómeros de propranolol em electrocromatografia capilar (nanopartículas à base de ácido metacrílico e nanopartículas revestidas com poliacrilamida). Para evitar interferência das nanopartículas com a detecção UV a técnica de preenchimento parcial foi utilizada.

Com as nanopartículas à base de ácido metacrílico a separação dos enantiómeros de propranolol não foi alcançada. Interações fortes inespecíficas entre as nanopartículas e o propranolol impossibilitaram a separação. Para obter suspensões estáveis de nanopartículas à base de ácido metacrílico elevadas quantidades de acetonitrilo foram utilizadas, favorecendo interações eletrostáticas que contribuíram para o aumento das interações inespecíficas.

As nanopartículas revestidas com poliacrilamida apresentam suspensões estáveis com baixas quantidades de acetonitrilo, devido as propriedades hidrofílicas da poliacrilamida. A separação dos enantiómeros de propranolol foi alcançada com 40% de acetonitrilo. As interações inespecíficas foram um factor dominante na irreproducibilidade dos resultados. Com o tempo vários factores contribuem para a diminuição da reproducibilidade dos resultados como por exemplo: interações entre as nanopartículas e a parede do capilar e a evaporação do solvente orgânico devido ao design dos tubos de ensaio utilizados na electrocromatografia capilar.

As nanopartículas revestidas com poliacrilamida são mais adequadas para a separação dos enantiómeros de propranolol devido á elevada estabilidade de suspensões apresentada em diferentes condições.

Nanopartículas de gadolínio silanizadas foram testadas como fase pseudoestacionária em electrocromatografia capilar. Características como a não detecção em UV e a grande área de superfície tornam estas nanopartículas de grande interesse para a separação de proteínas. A hormona de crescimento e a lisozima foram as proteínas analisadas.

A hormona de crescimento apresenta picos mais retidos quando se aumenta os tempos de injeção das nanopartículas, mostrando que interações entre a proteína e as nanopartículas ocorrem.

A utilização destas nanopartículas como fase pseudoestacionária permitiu a detecção da lisozima que não é visível utilizando electroforese capilar convencional. A adsorção da lisozima à parede do capilar é evitada devido ao uso destas nanopartículas.

Abbreviations

AIBN	Azobisisobutyronitrile
ACN	Acetonitrile
CE	Capillary Electrophoresis
CEC	Capillary Electrochromatography
CGE	Capillary Gel Electrophoresis
CIEF	Capillary Isoelectric Focusing
CZE	Capillary Zone Electrophoresis
EOF	Electroosmotic Flow
hGH	Human Growth Hormone
MAA	Methacrylic acid
MBA	N,N'-methylene-bis-acrylamide
MES	2-(N-morpholino)ethanesulfonic acid
MIP	Molecularly Imprinted Polymer
PSP	Pseudostationary phase
PSP-CEC	Pseudostationary phase capillary electrochromatography
TRIM	Trimethylolpropane trimethacrylate

Keywords

Nanoparticles

Miniaturization

Capillary Electrophoresis

Capillary Electrochromatography

Pseudostationary phase

Molecularly Imprinted Polymer

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Introduction

1. Capillary Electrophoresis

Capillary electrophoresis (CE) is a separation technique that combines the principles of electromigration mechanisms with the instrumentation and automation concepts of chromatography.¹ One of the greatest advantages is its diverse application range. CE is an attractive separation method because of its low sample and electrolyte consumption, short analysis time, high efficiency, ease of operation and automation compared with classical gel electrophoresis.²

1.1 Principles of Capillary Electrophoresis

1.1.1 Electrophoretic Mobility

Separation by electrophoresis is based on differences in solute velocity in an electric field.¹ The migration velocity v of an ion (Equation 1) is equal to the product of the field strength E (Vcm^{-1}) and the electrophoretic mobility μ_e ($\text{cm}^2\text{V}^{-1}\text{s}^{-1}$).

$$v = \mu_e E \quad (\text{Equation 1})$$

The electric field strength is a function of the applied voltage divided by the total capillary length. Ions can be separated when their charge-to-size rate (q/r_s) corresponds to different electrophoretic mobility values.

The electrophoretic mobility depends on the temperature and on the composition of the electrolyte solution.³

This equation is only useful for determining the apparent mobility. To calculate the actual mobility, the phenomenon of electroosmotic flow (EOF) must be accounted for. To perform reproducible electrophoresis, the EOF must be carefully controlled.⁴

1.1.2 Electroosmotic Flow

A fundamental component of CE operation is the EOF. This phenomenon is a consequence of the motion of an electrolyte solution under the influence of an electric field that results from the charge imbalance at a solid-liquid interface (Figure 1). Typically used for separations in CE a fused-silica capillary column is negatively charged in contact with an electrolyte solution with a $\text{pH} > 2-3$, due to the ionization of the surface silanol groups (Si-OH).⁴

Buffer cations build up near the surface to maintain charge balance, form the double-layer and create a potential difference next to the wall. This is known as the zeta potential. Some of these cations are firmly held to the region of the double-layer next to the capillary wall (the compact or Stern layer). The thickness of the electrical double-layer is small, typically 10nm.^{1,5}

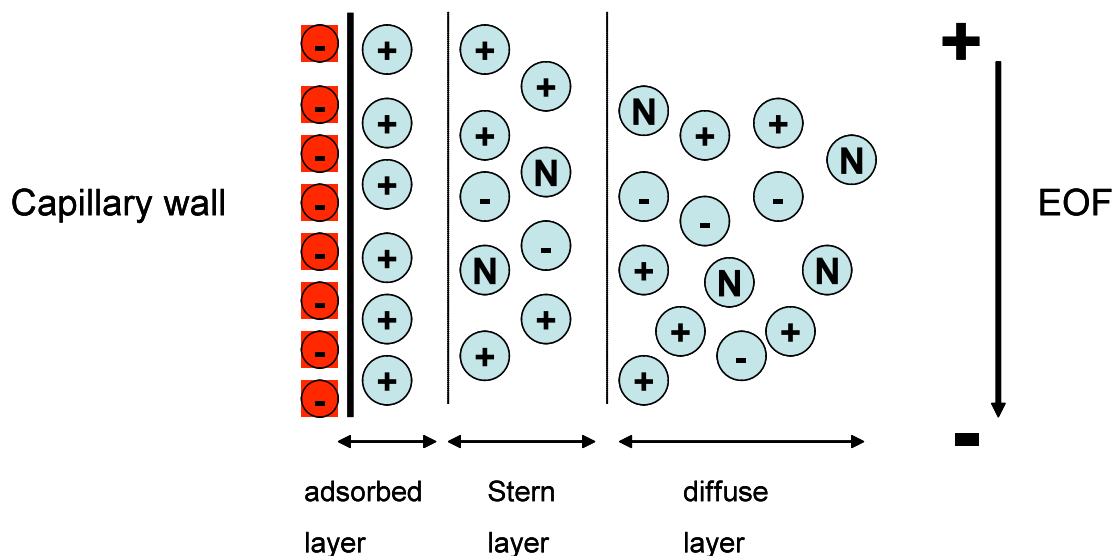


Figure 1-Representation of the double layer at the surface of the inner wall of fused-silica capillary column.

When an electric field is applied across the capillary the cations in the diffuse outer layer of the double-layer will be attracted toward the cathode and drag the bulk solvent along with them. Even though analytes migrate according to their charges within the capillary, the EOF is sufficient to sweep all positive, neutral and even negative species toward the same end of the capillary.³

A unique feature of EOF in the capillary is the flat profile of the flow (Figure 2). Since the driving force of the flow is uniformly distributed along the capillary there is no pressure drop within the capillary, and the flow is nearly uniform throughout.¹



Figure 2- Flow profile.

The electroosmotic flow can be expressed in terms of velocity or mobility by the equations (2) and (3):

$$v_{EOF} = (\epsilon\zeta / \eta)E \quad \text{(Equation 2)}$$

$$\mu_{EOF} = (\epsilon\zeta / \eta) \quad \text{(Equation 3)}$$

Where v_{EOF} = velocity

μ_{EOF} = EOF mobility

ζ = zeta potencial

ϵ = dielectric constant

The EOF often needs to be controlled and altered. There are several methods to accomplish this, as detailed in Table 1.¹

Table 1-Methods to control electroosmotic flow.

Variable	Result	Advantages/Disadvantages
Electric field	Changes rate of EOF	When lowered, efficiency and resolution may decrease Joule heating may result when increased
Buffer pH	EOF increase at high pH EOF decrease at low pH	Can effect the solute charge and mobility
Ionic strength	High ionic strength decreases EOF Low ionic strength increases EOF	High ionic strength generates high current an possible Joule heating Low ionic strength problematic for sample adsorption
Temperature	Changes viscosity	Useful since temperature is controlled instrumentally
Organic Modifier	Changes zeta potential and viscosity	Usually decreases EOF May alter selectivity
Surfactant	Adsorbs to capillary wall via hydrophobic and/or ionic interactions	Anionic surfactants can increase EOF Cationic surfactant can decrease EOF
Neutral hydrophilic polymer	Adsorbs to capillary wall via hydrophobic interactions	Decreases EOF by shielding surface charge and increasing viscosity
Covalent coating	Chemical bonding to capillary wall	Many modifications possible (hydrophilicity or charge) Stability often problematic

1.1.3 Resolution

Resolution in CE is the same as that for chromatography and is simply defined as:

$$R = \frac{2(t_2 - t_1)}{w_1 + w_2} = \frac{t_2 - t_1}{4\sigma} \quad (\text{Equation 4})$$

Where t = migration time

w = baseline peak width (in time)

σ = peak standard deviation

1 and 2 refer to the two solutes

The equation 4 describes the separation in terms of differential migration and the dispersive processes acting against it.^{1,3}

1.1.4 Dispersion

The uniformity of the flow profile (Figure 2) is of great importance in capillary electrophoresis to obtain high separation efficiencies. It is important to control dispersion because it can increase zone length and the mobility difference necessary to achieve separation. Dispersion effects due to flow non-uniformity are absent in capillary electrophoresis, so the separation efficiency is determined only by longitudinal diffusion and can be expressed by the following equation^{1,3}:

$$N = \frac{\mu_e E l}{2D} \quad (\text{Equation 5})$$

Where N=theoretical plates

μ_e =electrophoretic mobility of the analyte

E=applied electric field

l=effective capillary length

D= diffusion coefficient of the solute

The equation 5 shows that it is preferable to apply high fields because the solute will spend less time in the capillary and has less time to diffuse.

From an electropherogram it is possible to determinate directly the theoretical plate number using (Equation 6) ^{1,3}:

$$N = 5,54 \left(\frac{t}{w_{1/2}} \right)^2 \quad \text{(Equation 6)}$$

Where t=migration time of the analyte

$w_{1/2}$ = temporal peak width at half height

1.2 Instrumental aspects

One key feature of CE is the overall simplicity of the instrumentation (Figure 3). The ends of the capillary are placed in separated reservoirs, each containing an electrode used to make electrical contact between the high voltage power supply and capillary. The sample is injected onto the capillary by replacing one of the buffer reservoirs (normally at the anode) with a sample reservoir and applying either an electric potential or external pressure for a few seconds.⁴

The separation is performed when an electric potential is applied across the capillary. Optical detection of the separated analytes can be achieved directly through the capillary wall near the opposite end (normally near the cathode).¹

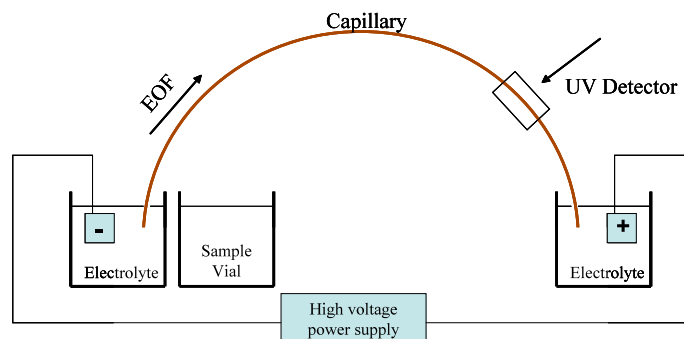


Figure 3-Schematic of CE instrumentation.

1.2.1 Capillary

The capillary material should ideally fulfill requirements like being chemical and electrical inert, UV-visible transparent, flexible, robust and inexpensive. For this reason fused silica material is the most employed in capillary electrophoresis. A detector window is prepared easily by removing the polyimide coating to leave a transparent section of about 1-2 mm long at a fixed distance from the column outlet. The capillary ends should be cut square and free of debris since misshapen capillaries ends can result in distorted peak shapes and poor quantitative precision.³

Fused silica-capillaries with internal diameters ranging from 25-75 μm with a range of outer diameters between 350-400 μm are usually used in CE. From an analysis time perspective, capillaries as short as possible should be used. Most commonly effective lengths between 50-75 cm are employed.⁴

1.2.2 Sample injection

The total capillary volume is in the microliter range, as a result of the small dimensions of the capillary. Adhering to the chromatography rule of thumb, the sample plug length should be less than 1 to 2% of the total length of the capillary.¹

Quantitative sample injection can be accomplished by a number of methods. The two most common are hydrodynamic and electrokinetic. In either case, the sample volume loaded is generally not a known quantity, although it can be calculated. Instead of volume, the quantifiable parameters are pressure/time for hydrodynamic injection, or voltage/time for electrokinetic injection.³

1.2.2.1 Hydrodynamic injection

Hydrodynamic injection can be accomplished by application of pressure at the injection end of the capillary, vacuum at the exit end of the capillary (Figure 4), or by siphoning action obtained by elevating the injection reservoir relative to the exit reservoir (Figure 5).³

The volume injected, V , can be calculated using the Hagen-Poiseuille equation (7):

$$V = \frac{\Delta P d^4 \pi t}{128 \eta L} \quad (\text{Equation 7})$$

Where ΔP = pressure difference across the capillary

d = capillary inside diameter

t = time

η = buffer viscosity

L = total capillary length

For siphoning injection, the pressure differential for use in last equation is given by equation 8:

$$\Delta P = \rho g \Delta h \quad (\text{Equation 8})$$

Where ρ = buffer viscosity

g = gravitational constant

Δh = height differential of the reservoirs

A typical siphoning injection is obtained by raising the sample reservoir 5 to 10 cm relative to the exit reservoir for 10 a 30 seconds, depending on the conditions. Siphoning is used in systems without pressure injection capabilities.¹

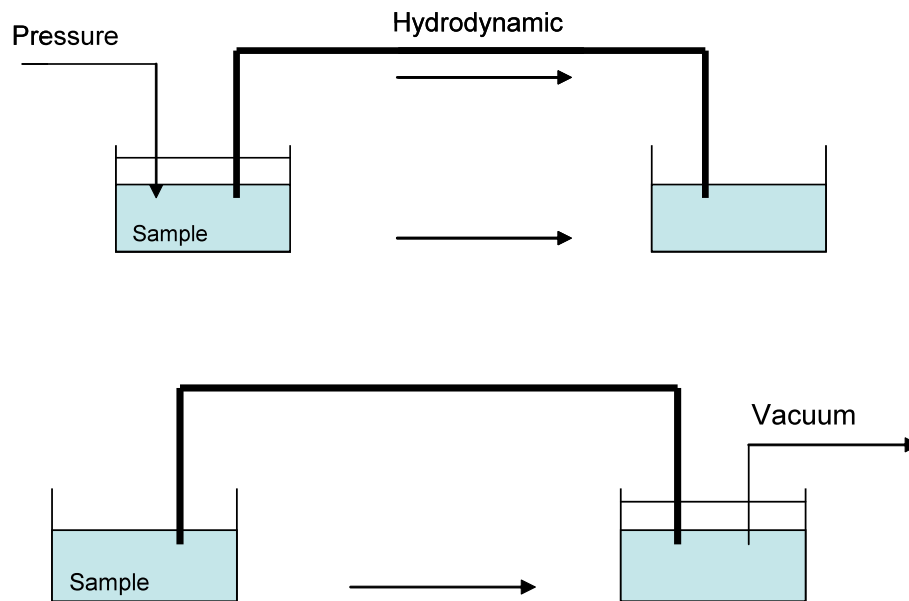


Figure 4-Hydrodynamic injection.



Figure 5-Siphoning injection.

1.2.2.2 Electrokinetic injection

Electrokinetic injection is performed by replacing the injection-end with the sample vial and applying the voltage (Figure 6). Sample enters the column by the combined effect of electroosmosis and electrophoresis. The quantity loaded is dependent on the electrophoretic mobility of the solutes and this result in discrimination with the more mobile sample components injected in greater quantities than the less mobile species.³

The quantity injected is dependent on the EOF, sample concentration, and sample mobility as described by the equation below:

$$Q = \frac{(\mu_e + \mu_{EOF})V\pi r_2 Ct}{L} \quad (\text{Equation 9})$$

Where μ = electrophoretic mobility of the analyte

μ_{EOF} = EOF mobility

V = voltage

r = capillary radius

C = analyte concentration

t = time

L = capillary total length

Electrokinetic injection is normally employed for capillary electrochromatography (CEC) and capillary gel electrophoresis (CGE) and when hydrodynamic injection is ineffective.¹

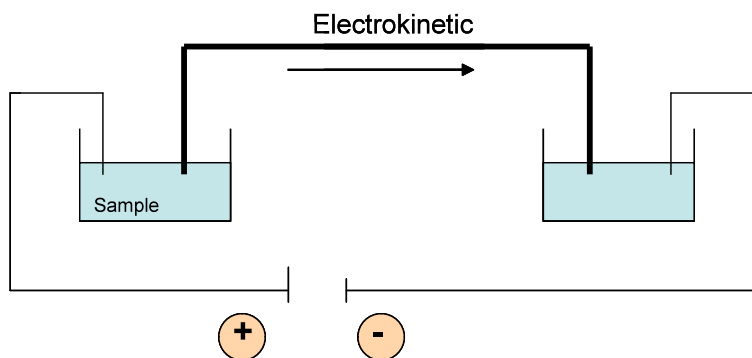


Figure 6-Electrokinetic injection.

1.2.3 Detectors

Detection in CE is a challenge as a result of the small capillary dimensions and their associated constrained injection volumes. Almost all capillary electrophoresis instruments are fitted with a UV-visible absorption detector as standard due to its universal detection nature.⁴ Other detection modes (Table 2) are available with fluorescence and conductivity detectors and interfaces to mass spectrometry as possible options.³

Table 2-Methods of detection.

<i>Detection Mode</i>	<i>Mass detection limit (moles)</i>	<i>Concentration detection limit (molar)</i>
<i>UV-visible</i>	10^{-13} - 10^{-15}	10^{-5} - 10^{-8}
<i>Fluorescence</i>	10^{-15} - 10^{-11}	10^{-7} - 10^{-9}
<i>Laser-induced fluorescence</i>	10^{-18} - 10^{-20}	10^{-14} - 10^{-16}
<i>Indirect</i>	10^{-15} - 10^{-17}	10^{-5} - 10^{-7}
<i>Amperometry</i>	10^{-18} - 10^{-19}	10^{-10} - 10^{-11}
<i>Conductivity</i>	10^{-15} - 10^{-16}	10^{-7} - 10^{-8}
<i>Potentiometry</i>	-----	10^{-7} - 10^{-8}
<i>Mass Spectrometry</i>	10^{-16} - 10^{-17}	10^{-8} - 10^{-9}

1.2.3.1 UV-Visible detection

UV-visible absorption is the most used detection method as mentioned before. The high efficiency in CE is in part due to on-column detection. In this case a small section of the polyimide coating is removed from the exterior of the capillary by burning, dissolution or scraping. This section of the capillary serves as an optical window, which avoids zone broadening because of dead-volume or component mixing.³In fact separation is still occurring while in the detection window.

For adsorptive detectors the absorbance of a solute is dependent on the path length, concentration and molar absorptivity as defined in Beer's law (Equation 10):

$$A=bC\varepsilon \quad (\text{Equation 10})$$

So the sensitivity of the on-column detector is critical due to the short effective path length, which restricts detection limits in concentration terms resulting in a relatively weak adsorption signal.¹

In order to improve the sensitivity of absorbance, several modifications of the detection cell have been suggested for increasing the path length of the measurements such as bubble cell, Z-cell and multireflection cell (Figure 7).⁴

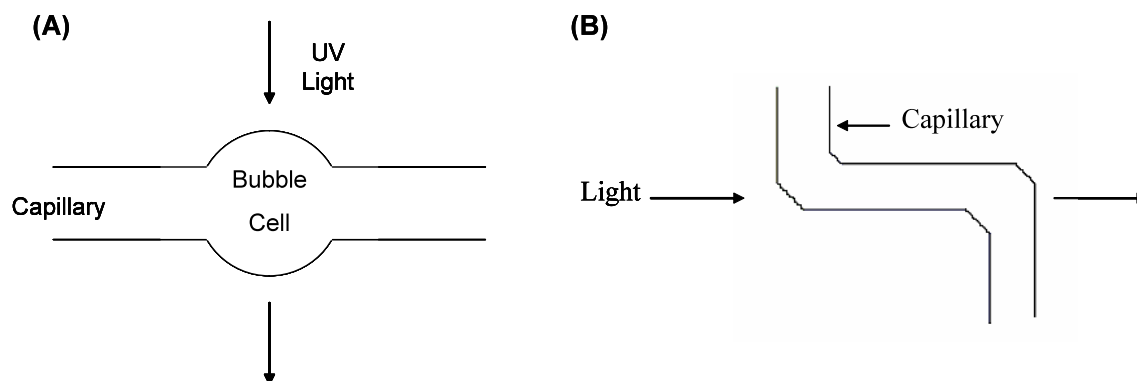


Figure 7-(A) Bubble cell, (B) Multireflection cell.

1.2.3.2 Fluorescence

Fluorescence is the most sensitive detection technique for capillary electrophoresis and especially laser-induced fluorescence (LIF). Lasers have a number of advantages: the collimated beam is easily focused into a small volume; excitation power can be varied over a wide range; and the source is inherently monochromatic. On the other hand lasers are more expensive, have a shorter operating life, and provide only a limited selection of operating wavelengths, particularly in the low UV region. Another concern with fluorescence methods is that sample usually requires labeling.^{3,4}

1.2.3.3 Indirect Detection

To detect species with low molar absorptivities, indirect detection can be applied. Detection is accomplished by placing an ionic chromophore in the electrophoresis buffer. The detector then receives a constant signal due to the presence of this substance. The analyte displaces some of these ions so that the detector signal decreases during the passage of the analyte band through the detector. The analyte is then determined from the decrease in absorbance.^{3,4}

1.2.3.4 Electrochemical

Electrochemical detection based in conductometry, amperometry or potentiometry is used to only a limited extent. Conductivity is a universal method for the detection of ions. Amperometry is a selective method for the detection of electroactive species by oxidation or reduction at an electrode surface. Potenciometry is a near –specific method for characteristic ions. One of the problems in separation is when high voltage is applied because it can lead to serious interference with the electrochemical signal. So isolation of the detector electrodes is required. This can be accomplished by inserting a porous glass or graphite joint between the end of the capillary and a second capillary containing the detector electrodes.^{3,4}

1.2.3.5 Mass spectrometry

Capillary electrophoresis with mass-spectrometric detection has been used for the determination of large biomolecules, such as proteins, DNA fragments, and peptides. It is a sensitive method that offers structural information. The most common sample introduction/ionization interface used is electrospray. One disadvantage is the complex interface between CE and MS.^{3,4}

1.3 Modes of operation

1.3.1 Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) is the most used mode due to the diverse application range and its simplicity and versatility of operation.

In CZE the buffer composition is constant throughout the region of the separation.⁴ Separation occurs because solutes migrate in discrete zones and at different velocities (Figure 8). Due to the EOF it is possible to separate both anionic and cationic solutes in CZE. Neutral solutes do not migrate and all coelute with the EOF. CZE is very useful for the separation of peptides and proteins.¹

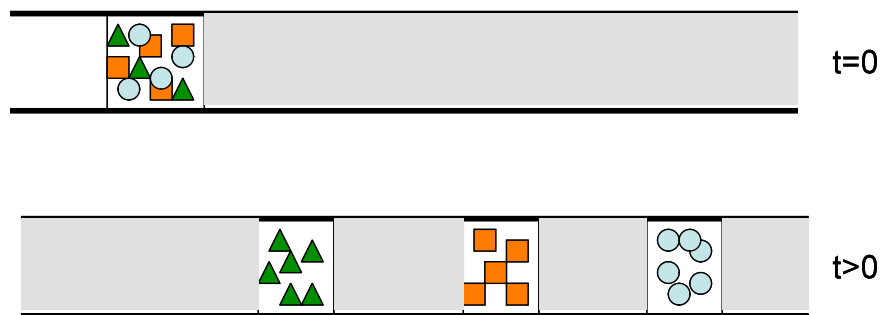


Figure 8-Separation in CZE.

1.3.2 Capillary isoelectric focusing

Capillary isoelectric focusing (IEF) is used to separate peptides and proteins on the basis of pI (Figure 9). A pH gradient is formed within the capillary using ampholytes. Ampholytes are molecules that contain both an acidic and a basic moiety and can have pI values that span the desired pH range of the IEF experiment.

After filling the capillary with a mixture of solute and ampholytes, the gradient is formed. With a basic solution at the cathode and an acidic solution at the anode, when the potential is applied the charged ampholytes and proteins migrate through the medium

until they reach a region where they are becoming uncharged (at their pI). This process is known as focusing.⁴

In order to detect the focused bands it is necessary to mobilize the contents of the capillary so that the bands pass the detector. Mobilization can be accomplished through application of pressure to the capillary or by addition of salt to one of the reservoirs.

In this technique the EOF should be reduced or suppressed since the flow could flush the ampholytes from the capillary before focusing is completed. This can be accomplished by the use of dynamic or covalent coatings.³

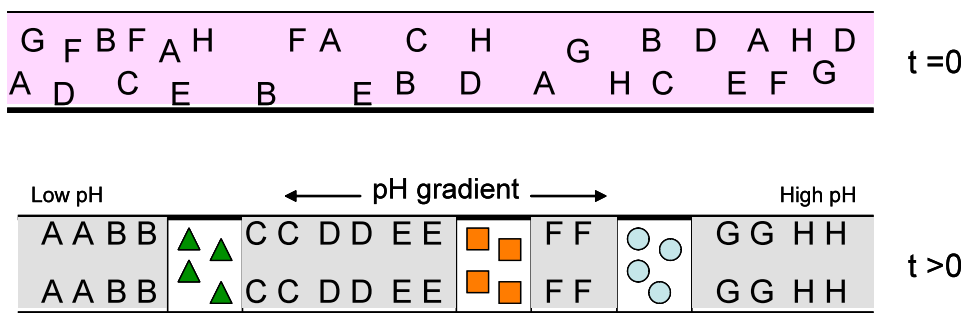


Figure 9-Separation in IEF.

1.3.3 Capillary Isotachopheresis

In capillary isotachopheresis (ITP) a state is created in which separated zones migrate all at the same velocity due to the combination of two buffer systems (Figure 10). The zones remain sandwiched between so-called leading and terminating electrolytes. The leading one contains ions of a higher mobility than any of the analyte ions and a terminating one with ions of a lower mobility than the sample ions. In any particular application only anions and cations can be separated, but not both at the same time.³

The steady-state velocity in ITP occurs since the electric field varies in each zone. The field is self-adjusting to maintain constant velocity, with the lowest field across the zone with the highest mobility. The boundary between zones is very sharp because of this phenomenon. If a solute species starts to diffuse into the next zone, it encounters a lower field, which changes velocity and it immediately returns to its zone.

An interesting feature of ITP is the constant concentration in each zone, determined by the concentration of the leading electrolyte. Zones that are less concentrated than the leading electrolyte are sharpened to adapt to the proper concentration.⁴

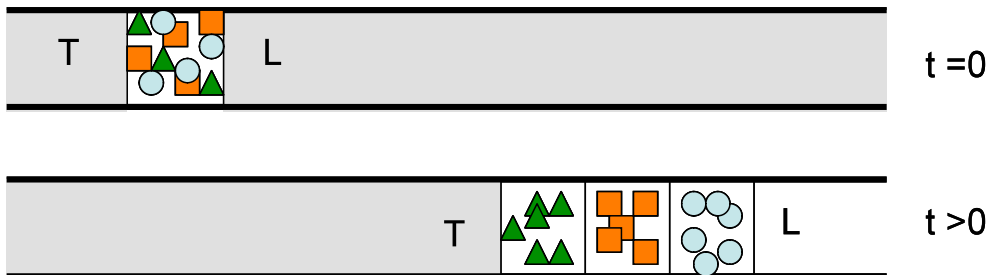


Figure 10- Separation in ITP.

1.3.4 Capillary gel electrophoresis

Capillary gel electrophoresis (CGE) is comparable to traditional slab or tube gel electrophoresis since the mechanisms are identical. This technique is helpful in the separation of macromolecules such as proteins and nucleic acids.

CGE is performed in a porous gel polymer matrix that provides a molecular sieving action that retarded the migration of analytes depending of the pore size of the polymer and the size of the analyte ions (Figure 11). As charged solutes migrate through the polymer the larger solutes will be delayed more than smaller ones.^{3,4}

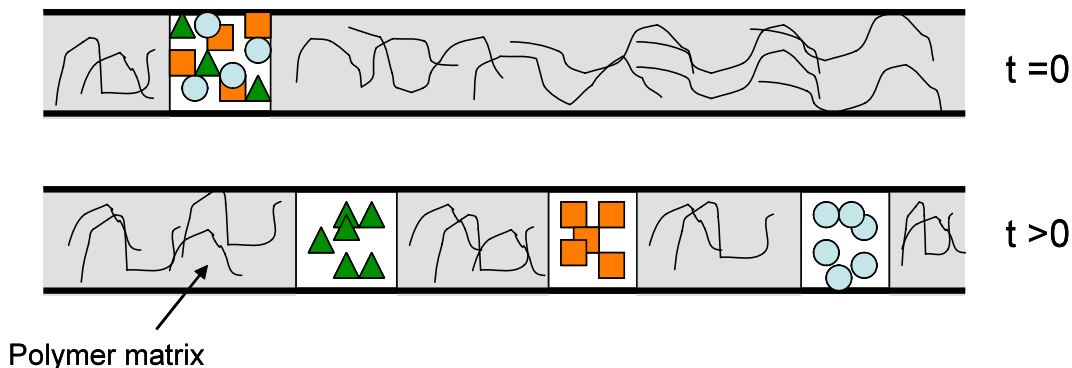


Figure 11-Separation in CGE.

1.3.5 Micellar electrokinetic chromatography

Micellar electrokinetic chromatography (MEKC) is an electrophoretic technique that can be used for the separation of neutral solutes as well as charged ones. This technique involved introduction of surfactants in the running buffer at concentration levels above the critical micelle concentration, at which micelles form (Figure 12).¹ Micelles are spherical with the hydrophobic tails of the surfactant orientated to the center and the charged heads oriented toward the buffer.

Surfactants can be anionic, cationic, non-ionic, zwitterionic, or mixtures of each. Depending on the charge of the micelles they migrate either with or against the EOF.⁴

When the sample is introduced into the system, micelles can interact in a chromatographic way through both hydrophobic and electrostatic interactions (the components distribute between aqueous phase and the hydrocarbon phase in the interior of the micelles). For neutral species, it is only partitioning in and out of the micelle that effects the separation. Longer migration times are expected to the more hydrophobic compounds that interact strongly with the micelle.

The direction of solute and micelle migration varies and depends on the micelle charge and the rate of EOF.³

MEKC is a dynamic mode that can be used in a wide range of substances with hydrophilic and hydrophobic characteristics. Applications include amino acids, nucleotides, vitamins, aromatic hydrocarbons, to name a few.¹

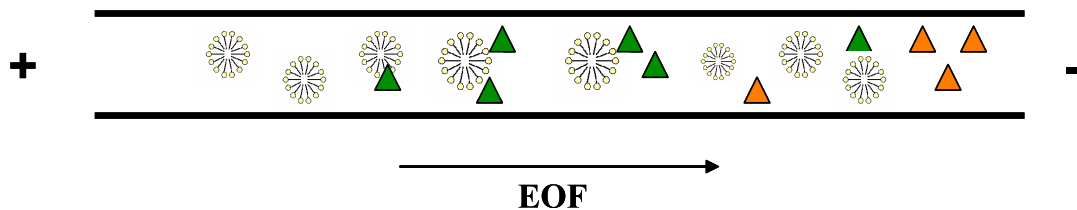


Figure 12-Separation in MEKC. Distribution of analytes based in their hydrophobicity.

1.3.6 Capillary electrochromatography

Electrochromatography was first introduced more than 32 years ago by Pretorius⁶ who demonstrated the feasibility of performing electrodriven separations by applying an electric field across a glass chromatographic column packed with microparticulate silica. However, due to poor heat dissipation in columns with large internal diameters, only weak electrical fields could be used, resulting in slow EOF and significantly slower analysis. Jorgenson and Lukacs⁷ demonstrated the concept of electrochromatography using capillary columns packed with silica-based particles. The revival of capillary electrochromatography (CEC) occurred in the 90s as a result of the need for new miniaturized separation methods with vastly enhanced efficiency and peak capacity.^{8,9}

In electrochromatography, a mobile phase is transported through a stationary phase by electroosmotic flow pumping rather than mechanical pumping.³ So the stationary phase in CEC plays a dual role because it provides sites for the required interactions with analytes and it also drives EOF.¹⁰

In principle, CEC enables the exploitation of a plug-like flow profile to achieve high efficiency, while retaining the variety of retention mechanisms and selectivity afforded by HPLC. Separations of uncharged solutes are affected by differential interactions of the solute between the mobile and the stationary phases. Charged species on the other hand can be influenced by the applied electric field and by interactions with the stationary phase; hence separation of charged species is a combination of both differential interaction with the stationary phase and differential electromigration.^{11,12}

CEC can be carried out in different modes. Generally CEC is performed using packed⁷, monolith¹³, open tubular¹⁴ columns. Another approach to performed CEC is the use of particles which are viewed as a pseudostacionary phase (PSP).¹⁵

A packed column is a capillary filled with particles that are confined between two end-frits. An open-tubular column is a capillary bonded with a wall-supported stationary phase that can be a coated polymer, bonded molecular monolayer, or a synthesized porous layer network. A monolith column is a capillary containing a wall-supported porous continuous bed that is formed *in situ* in the capillary.¹⁶

1.3.6.1 Packed columns

The use of packed capillaries columns in CEC has already been demonstrated in numerous reports.¹⁷ The preparation of a packed capillary involves the selection of packing materials; column packing and the preparation of end-frits. Packing materials for CEC should have both charged groups to provide EOF and groups to provide separation selectivity.¹⁶ The preparation of these columns includes two key steps: the packing of small diameter particles into narrow-bore tubes and the fabrication of retaining frits within the capillary.¹⁰ The frits are necessary to prevent packing material from migrating out of the capillary by electrophoresis, EOF, or pressure. One benefit of having column frits is the elimination of unions and connective tubing between the column and injector, and between the column and detection cell. This results in minimization of column band dispersion and contributes to improved peak efficiency along with the plug-like velocity profile generated by EOF.¹⁸ Although packing is currently a well-established technique for the production of CEC columns, repeatability of this procedure remains problematic.¹⁰ Another disadvantage of packed capillaries is the need of the preparation of frits by sintering a zone of the packing material. Such frits contribute to column band spreading, bubble formation, and column fragility.^{19, 20}

1.3.6.2 Monolith columns

Monolith columns are continuous porous rods of stationary phase inside the column. Due to their unique properties, monolithic columns have attracted considerable attention. The most appealing aspect of monoliths is the ease of their *in situ* preparation that avoids problems related to both frit fabrication and packing.^{10, 21} In addition, the polymerization mixture used for the preparation of organic polymer monoliths can be prepared from a wide variety of precursors, allowing a nearly unlimited choice of both matrix and surface

chemistries.¹³ The control that can be exerted over the preparation process facilitates optimization of the porous properties of the monolith, and consequently the chromatographic efficiency of the entire system.¹⁰

Despite the success of monolith CEC columns, a more complete understanding of the polymerization mechanism is needed to control morphology of the monoliths since large porosity is necessary to provide convective flow through the stationary phase.²²

1.3.6.3 Open-tubular columns

The open tubular CEC format is characterized by the preparation of the stationary phase as a thin layer on the inner wall of the capillary. The first report on using open tubular CEC was by Tsuda et al. in 1982.²³ They succeeded in separating aromatic compounds by CEC with acetonitrile and water as a mobile phase. Since then several different methods to prepare open-tubular columns for CEC have been developed.²⁴

It has several advantages compared with the other modes, no bubble formation problems because end-frits are not required, small internal diameter columns are used, and the stationary phase is homogeneous. The column length can also be easily shortened.¹⁶ However an important limitation of this approach, is the low sample load capacity due to the low quantity of polymeric material available in the capillary.

1.3.6.4 Particles as a Pseudostationary Phase

Another way to perform CEC is to use a pseudostationary phase (PSP). PSPs are interaction phases that are moving with (or against) the mobile phase. The first PSP that was used in an electrochromatography system was micelles by Terabe et al.²⁵ Micelles have the drawbacks of limited stability, sensitivity to organic solvents and problems when used in conjunction with MS. A way to solve the problems with micelle is to use nanoparticles as PSP, which was introduced in 1989 by Wallingford and Ewing.²⁶

Nanoparticles are defined as monomolecular particles, whose sizes are measured in nanometers and they should fulfill some important characteristic features to be suitable in PSP-CEC²⁷:

- Form homogeneous dispersions in a wide range of electrolytes;
- Selectivity in the interaction with the analytes;
- Charged, to prevent coelution with EOF;
- Equal mobility to prevent peak broadening;
- Small mass transfer resistance;
- Not disturb detection;
- Small to provide high surface area for the analytes and to avoid light scattering.

The introduction and characterization of novel nanoparticles has been motivated by the desire for enhanced separation performance, unique selectivity, improved capability for selected applications, or compatibility with specific detectors. Different nanoparticles can be used as PSP in CEC as described in Table 3.¹⁵

Table 3-Applications of different nanoparticles in PSP-CEC.

PSP	Analytes
Silica nanoparticles Surfactant stabilized RP particles (1500 nm) Surfactant stabilized RP particles (500 nm) Chemically modified silica particles (500 nm) Colloidal silica Organically modified silica	Phenol derivatives ²⁸ Polyaromatic hydrocarbons (PAHs) ²⁹ PAHs and naphthalene derivatives ³⁰ Dialkyl phthalates and steroids ³¹ Aromatic acids ³²
Gold nanoparticles Gold nanoparticles modified with poly(ethylene oxide) Gold nanoparticles modified with poly(ethylene oxide)	Long dsDNA ³³ Long dsDNA ³⁴
Polymers nanoparticles Sulfonated hydrophobic nanoparticles (20 nm) Polymer particles Sulfonated polystyrene-divinylbenzene particles (225 nm) Nanoparticles formed by precipitation polymerization Polystyrene particles	Catechols ²⁶ Derivatized alkyl amines ³⁵ Alkali metals and ammonium ions ³⁶ Amines ³⁷ Propanolol ³⁸
Fullerenes	PAHs ^{39, 40}
Carbon nanotubes Carboxylic single-walled nanotubes (SWNTs) Multi-walled nanotubes	Theobromine Caffeine ⁴¹ Purine ⁴² pyrimidine

The main advantages of this PSP system are one-time use of the stationary phase allowing fast regeneration of column, absence of stationary phase carry-over effects, easily exchanged column and either retaining frits or complicated packed procedure is necessary.¹⁵ The main drawback in nanoparticle-based PSP-CEC is the negative effect of nanoparticles on detection.⁴³ A way to solve this problem has been to use partial filling technique^{44, 45} or continuous full filling technique^{37, 46}.

1.3.6.4.1 Partial filling technique

Due to the light scattering properties of the nanoparticles the partial filling^{47, 48} was developed to avoid disturbance on UV detection. It was first described by Valtcheva et al.⁴⁴. Using partial filling a plug of PSP is injected prior to (or subsequent to) the analytes (Figure 13). This allows the analytes and the PSP to enter the detector at different times which minimizes interference during detection of the analytes.³⁷ Optimization of partial filling technique might be time-consuming due to co-elution of the analytes and PSP.⁴³

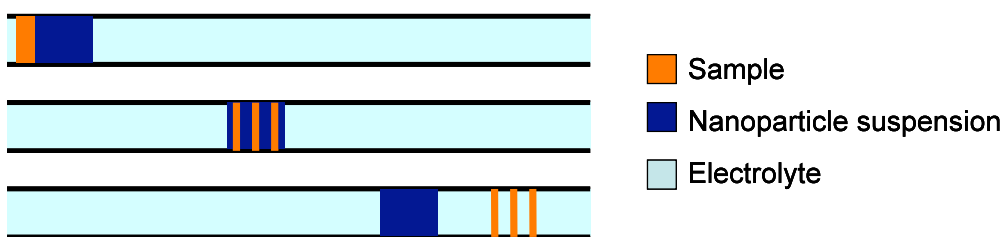


Figure 13-Partial filling technique.

1.3.6.4.2 Continuous full filling technique

In the continuous full filling technique the nanoparticles are suspended in the entire electrolyte, which then is continuously introduced during the separation (Figure 14).^{37, 46} The continuous full filling technique requires the possibility to detect analytes in the presence of the PSP, or separation of the PSP and analytes prior to detection.⁴³

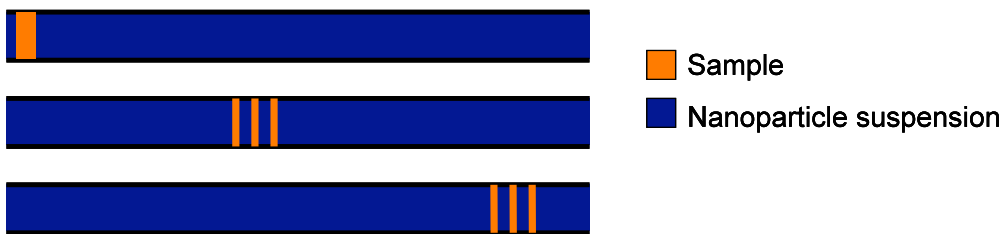


Figure 14-Continuous full filling technique.

1.4 Molecular Imprinting

Molecular imprinting is a technique used for the preparation of polymeric matrices with selective molecular recognition capability towards a predetermined ligand. This method can be performed in two different ways either covalent⁴⁹ or non covalent⁵⁰, depending on the nature of adducts between functional monomer and template.

Molecular imprinting processes are composed of the following three steps (Figure 15):

- Preparation of covalent conjugate or non-covalent adduct between a functional monomer and a template molecule;
- Polymerization of this monomer-template conjugate (or adduct);
- Removal of the template from the polymer.⁵¹

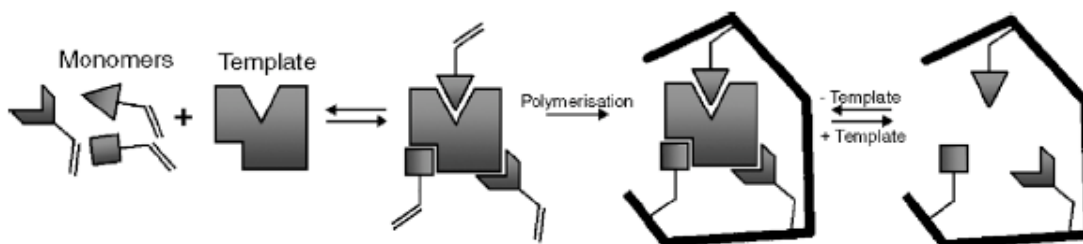


Figure 15-Schematic of the molecular imprinting process.

In step 1, functional monomer and template are connected by a covalent linkage (covalent imprinting) or they are placed nearby through non-covalent interactions (non-covalent imprinting). In step 2, the structures of these conjugates (or adducts) are frozen in a three-dimensional network of polymers. The functional residues (derived from the functional monomers) are topographically complementary to the template. The choice of the template defined selective affinity of an analyte to the molecularly imprinted polymer.

Finally in step 3 the templates molecules are removed from the polymer. Here the space in the polymer originally occupied by the template molecule is left as a cavity. Under appropriate conditions, these cavities satisfactorily remember the size, structure, and other physicochemical properties of the template, and bind this molecule (or its analog) efficiently and selectively.

The choice of the best method strongly depends on various factors and both have advantages and disadvantages as described in Table 4.

Table 4- Advantages and disadvantages of covalent and non-covalent imprinting.

	Covalent	Non-covalent
Synthesis of monomer-template conjugate	necessary	unnecessary
Polymerization conditions	rather free	restricted
Removal of template after polymerization	difficult	less difficult
Guest-binding and guest-release	slow	fast
Structure of guest-binding site	clearer	less clear

Due to their favorable molecular recognition capability and stability, potential applications of molecular imprinting polymers (MIPs) have been investigated in broad areas, such as ligand binding assays, liquid chromatography, solid-phase extraction, sensors, and catalytic chemical reactions.⁵²

Before, MIPs were synthesized in the form of monoliths, which after grinding and sieving, gave irregular particles with different sizes. The grinding and sieving process is time-consuming and yields only moderate amounts of useful imprinted polymers. The irregularity of size and shape of the obtained particles is not ideal for chromatographic purposes. To satisfy different analytical applications MIPs with well controlled physical forms in different size ranges are highly desirable. In addition to improve binding performance of MIPs, new physical formats of MIPs and more efficient synthetic methodologies were proposed. A simple method to prepare MIPs is precipitation polymerization method.^{45, 53} In this technique, polymeric spherical particles precipitate as they reach their solubility limit due to their increasing size.^{37, 54}

1.5 Molecular Imprinting formats for capillary electrochromatography

The high selectivity and stability of the MIP make it an interesting candidate for application as stationary phase sorbent in chromatography.

The use of CEC was a way to improve the efficiency of the MIP-based separation system. The miniaturized features of the capillary column allows the synthesise of a MIP stationary phase with low amounts of monomers, templates and solvents.^{22, 55} In this context miniaturized separation formats are gaining importance.²²

MIP formats for CEC can be divided in different categories, the monolith format, the packed format, the open tubular format and the nanoparticle format.

1.5.1 MIPs for open tubular CEC

The preparation of MIP open tubular capillaries offers advantages in that the *in situ* preparation is simple, and the efficiency in electrochromatographic separations is high.²² In open tubular CEC, thin films of MIPs are bonded to the internal wall of the capillary. Normally the imprint molecule is the more strongly retained and is therefore eluted last.⁵⁶ However an important limitation of this approach is the low sample load capacity due to the low quantity of polymeric material available in the capillary. The thickness of the molecular imprinted capillary coatings should be large enough in order to accommodate the stereoselective binding sites, but small enough to provide a rapid transfer of solutes from the electrolyte to the imprinted sites.

1.5.2 MIP packed capillaries

The preparation of MIP packed columns consists in the synthesis of the polymer in bulk, followed by grinding and sieving to obtain particles with a diameter of less than 10 μ m.^{57,}

58

The capillary showed enantiomer separation properties, with improved peak shape and resolution in the CEC system when compared with HPLC. The approach is however associated with serious limitations, the most important probably being the need of frits. In particular the use of acrylamide gel for this purpose may result in non-specific interactions with the analytes, leading to peak tailing and efficiency losses. The irregularly shaped particles and their broad size distribution, obtained by the crushing and sieving technique, further limit the efficiency of the system.

An important advance in this regard was the introduction of MIP-silica composites as new stationary phase material for CEC.²²

1.5.3 Monolith MIPs in CEC

Monoliths offer important advantages, particularly for MIP-CEC applications, due to the easy in situ preparation and the absence of retaining frits. However, they rely on the presence of large pores to provide convective flow through the stationary phase. In the case of MIPs, these pores must be generated simultaneously with the generation of the molecular scale binding sites, which constitutes the main limitation of this approach.²²

The main parameters controlling the pore size distribution are temperature, crosslinking level and the nature and content of porogenic solvent. Thus in thermally initiated polymerizations, large pores are promoted by low polymerizations temperatures, lower crosslinking levels and the use of poor solvents for the growing polymer chains. Successful formation of stable imprinted sites, on the other hand, is typically promoted by low polymerization temperatures, high crosslinking levels and aprotic poorly polar porogens.²²

1.5.4 MIP nanoparticles

MIP nanoparticles can be added to the background electrolyte which offers many attractive features. It avoids time-consuming capillary packing, frit formation and the method development can be fast, as conditions and type of MIP selector can be quickly changed prior to every run.^{22, 59}

The nanoparticles, with sizes ranging between 100 and 600nm, are too small to be efficiently packed in a capillary column. Using the partial-filling technique the use of this particle-based phase is facilitated.^{55, 60}

Despite the simplicity and attractive features of the set up, the filling techniques also have limitations.²²

2. Material and Methods

2.1 Chemicals

Trimethylolpropane trimethacrylate (TRIM, technical grade) was obtained from Aldrich (Dorset, UK) and used as received. Acetonitrile (ACN, 99.7%) and azobisisobutyronitrile (AIBN, 98%) used for nanoparticle synthesis were purchased from Merck (Darmstadt, Germany). AIBN was re-crystallized from methanol before use. Methacrylic acid (MAA, 98.5%) was purchased from ACROS (Geel, Belgium) and used as received. Acrylamide (>99.9 %) was purchased from Bio-Rad (Hercules, CA) and used as received. N,N'-methylene-bis-acrylamide (MBA, $\geq 98.0\%$) was purchased from Sigma (Gillingham, UK). (R)-propranolol hydrochloride (99%) supplied by Fluka (Dorset, UK) was converted into free base form before use.

Water was purified by a MiliQ purification system (Millipore, Bedford, MA, USA). Tricine, 2-(N-morpholino)ethanesulfonic acid (MES) and formic acid were obtained from Sigma (St. Louis, MO), sodium hydroxide from Eka Nobel (Sweden). Acetic acid, ammonium formate, phosphoric acid (85 %), potassium phosphate, citric acid and ammonium acetate were obtained from Merck (Hohenbrunn, Germany).

2.2 Preparation of nanoparticles

2.2.1 MIP nanoparticles

2.2.1.1 Methacrylic acid based nanoparticles

The template molecule, (R)-propranolol in its free base form (137 mg, 0.53 mmol) was dissolved in 40 ml of acetonitrile in a 150 mm×25 mm borosilicate glass tube equipped with a screw cap. MAA (113 mg, 1.31 mmol), TRIM (684 mg, 2.02 mmol) and AIBN (28 mg, 3 wt% of monomer) were then added. The solution was purged with a gentle flow of argon for 5 min and sealed under argon. Polymerization was carried out by inserting the borosilicate glass tube in a water bath pre-set at 60 °C for 24 h. After polymerization, particles were collected by centrifugation at 18,000 rpm (38,000×g) for 20 min. The template was removed by batch-mode solvent extraction with methanol containing 10% acetic acid (v/v), until no template could be detected from the washing solvent by spectrometric measurement (UV 290 nm). Polymer particles were finally washed with acetone and dried in a vacuum chamber. The resulting MIP particles, MIP(R) were imprinted against (R)-propranolol.

2.2.1.2 Core shell MIP nanoparticles

Molecularly imprinted polymer core-shell particles were synthesized by dissolving 65 µl (0.77 mmol) MAA, 378 µl (1.2 mmol) TRIM, 9.3 mg AIBN (2 wt% relative to the monomers) and 81 mg (0.31 mmol) (R)-propranolol in 47 mL acetonitrile in a 50 mL three neck round-bottom flask. A magnetic stir bar was put in the flask. The solution was gently purged with nitrogen for 10 min.

In a dropping funnel with pressure equalizing arm, 59 mg (0.38 mmol) MBA, 175 mg (2.5 mmol) acrylamide and 4.7 mg AIBN was dissolved in 23 ml acetonitrile. The solution was gently purged with nitrogen for 10 min. The funnel was attached to the flask and the flask was mounted to a Dean-Stark receiver. The free opening of the flask was closed with a glass plug. The flask was submerged in an oil-bath and heated to 80°C

during 20 min under stirring. Then the oil-bath was heated to 115°C and the reaction system was kept on reflux for 60 min. The reaction temperature in the round bottom flask was 83.0-84.0°C. Then the acetonitrile was distilled out dropwise and at the same rate solution from the funnel was added. The reaction was stopped when 23 mL of acetonitrile was distilled out from the reaction system and the funnel was completely empty after about 1.5 hours. After the polymerization, the particles were collected by centrifugation. The template (propranolol) was extracted by washing with 30 mL acetic acid:methanol mixture (10:100, v/v) repeatedly, until no template could be detected from washing solvent by UV-visible spectrophotometer. Approximately seven times with over-night washing was needed. Particles were finally washed with acetone and dried in vacuum chamber.

2.2.2 Silanized gadolinium oxide nanoparticles

The protocol from these nanoparticles is confidential.

2.3 Capillary Electrophoresis and Capillary Electrochromatography

CE and CEC experiments were performed on a Beckman P/ACE System 5010 (Beckman, Fullerton, CA, US) equipped with a UV detector. Fused-silica capillary (50 µm i.d.; 375 µm o.d.) with 25 cm total length and 18 cm effective length were obtained from Polymicro Technologies (Phoenix, AZ, US).

The electrolytes used were either pure aqueous buffers or suspensions of nanoparticles suspended in the electrolyte. Different aqueous buffers in a wide range of concentrations and pH were used to performed experiences. The samples were prepared from stock solutions (Table 5) diluted with electrolyte to the desirable concentration.

Table 5- *Sample stock solutions.*

Sample	Concentration
Rac-propranolol	5mM
(R)-propranolol	10mM
(S)-propranolol	10mM
Lysozyme	1 mg/mL
Human growth hormone (hGH)	1 mg/mL

The capillary was rinsed with 1M sodium hydroxide (1min), water (1min), and electrolyte (1min) between consecutive runs. Samples were injected electrokinetically while nanoparticles were hydrodynamic injected. Prior to separation, all solutions and nanoparticles suspensions were degassed by sonication.

3. Results and Discussion

3.1 MIP-based CEC enantiomer separation

To avoid absorbance and scattered light from MIP nanoparticles using UV detection, the partial filling technique was used. This technique allows manipulation of the amount of MIP used for a certain separation. A new and fresh amount of MIP nanoparticles are used for each CEC run.

For analytes to reach the detector window prior to the MIP it is important to optimize the CEC system. The migration of the MIP nanoparticle is determined by EOF of the system and the charge of the MIP. In the present study, migration of MIP nanoparticles was mainly due to the EOF.

Enantiomer separation of propranolol (Figure 16) in R (+) and S (-) form was attempted with two different types of nanoparticles.

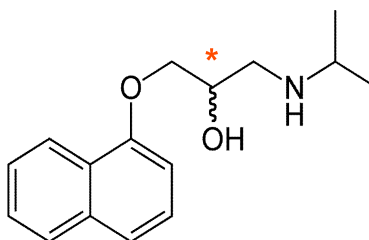


Figure 16-Chemical structure of propranolol. The asterisk indicates the chiral center of propranolol.

Methacrylic acid based nanoparticles and core shell nanoparticles were used as pseudostationary phase which will be described in the following sections.

3.1.1 Methacrylic acid based nanoparticles

MIP nanoparticles composed of a crosslinked MAA imprinted core were synthesized using R-propranolol as a template. Due to the characteristics of the polymers the nanoparticles are hydrophobic (Figure 17).

By dynamic light scattering (DLS) the average diameter of the nanoparticles was measured to 100 nm.

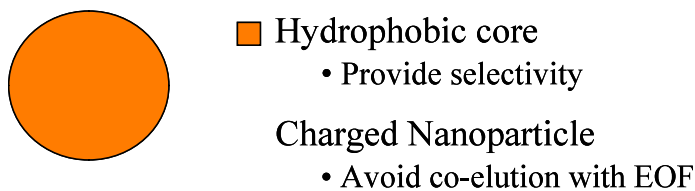


Figure 17-Nanoparticle schematic.

3.1.1.1 Suspension stability of nanoparticles in different electrolyte compositions

Electrolytes solutions with different buffer types, pH and ionic strength were studied in order to obtain stable suspensions of MIP nanoparticles (Table 6). The MIP nanoparticles were suspended in the electrolyte to a concentration of 5mg mL⁻¹.

Table 6- Composition of electrolyte tested. Nanoparticles sedimentated ☒, stable√.

ACN Content (%v/v)	Phosphate		Citrate		Formate		Acetate	Ammonium Acetate			MES	
	pH=2		pH=3,16		pH=3		pH=4	pH=6			pH=7	
	30 mM	50 mM	25 mM	20 mM	50 mM	20 mM	100 mM	2 M	20 mM	50 mM	100 mM	20 mM
90	☒	☒	☒	√	√	√	√	☒	√	√	√	√
70	☒	☒	☒	√	√	√	√	☒	√	√	√	√
50	☒	☒	☒	☒	☒	☒	☒	☒	☒	☒	☒	☒

Due to the hydrophobic properties of methacrylic acid based nanoparticles, higher contents of organic solvent, ACN were necessary to obtain suspension stability. The pH seems to have lower effect in suspension stability as seen in Table 6. Higher concentrations of buffer give instable suspensions of nanoparticles.

Therefore electrolyte compositions that give suspension stability of MIP nanoparticles were used in the following CEC studies.

3.1.1.2 CEC enantiomer separation of rac-propranolol

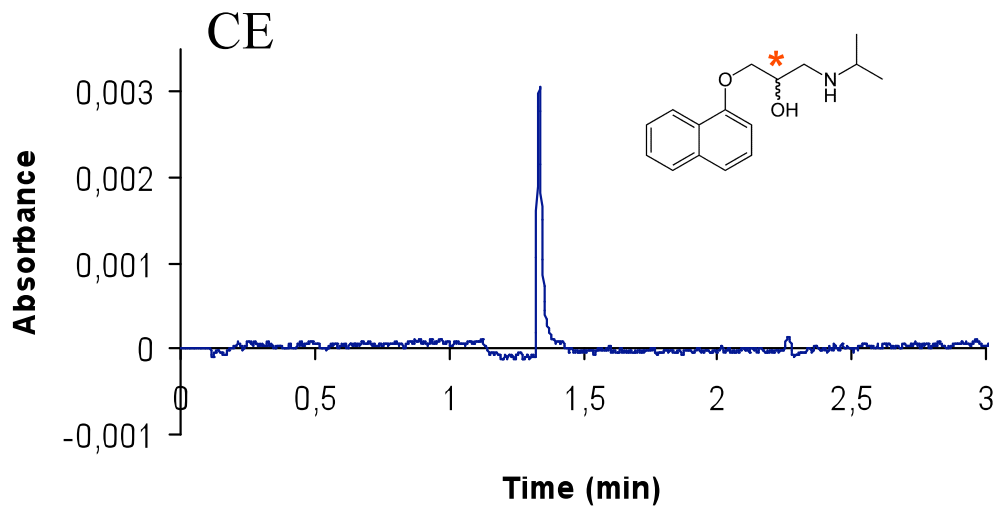
Enantiomer separation of propranolol was not achieved using any of the electrolytes compositions described in Table 6 (Figure 18, 19, 20). Different plugs lengths of the MIP nanoparticles and sample were examined.

Electrostatic interactions have a dominant effect on recognition when high levels of ACN are used. This can lead to unspecific interactions between the sample, propranolol and the MIP nanoparticles. Even though MIP nanoparticles present specific interaction for R-propranolol they are not strong enough to overcome the unspecific interactions in the conditions where MIP nanoparticles formed stable suspensions.

In a more aqueous environment the natural tendency of propranolol and MIP nanoparticles would be to interact with each other by van Der Waals interactions due to their hydrophobic characteristics. A decrease of organic solvent, ACN would improve separation but it was proved before that high levels are necessary for suspension stability of MIP nanoparticles.

The synthesis of MIP nanoparticles with a more hydrophilic characteristic would improve suspension stability and thereby separation.

(A)



(B)

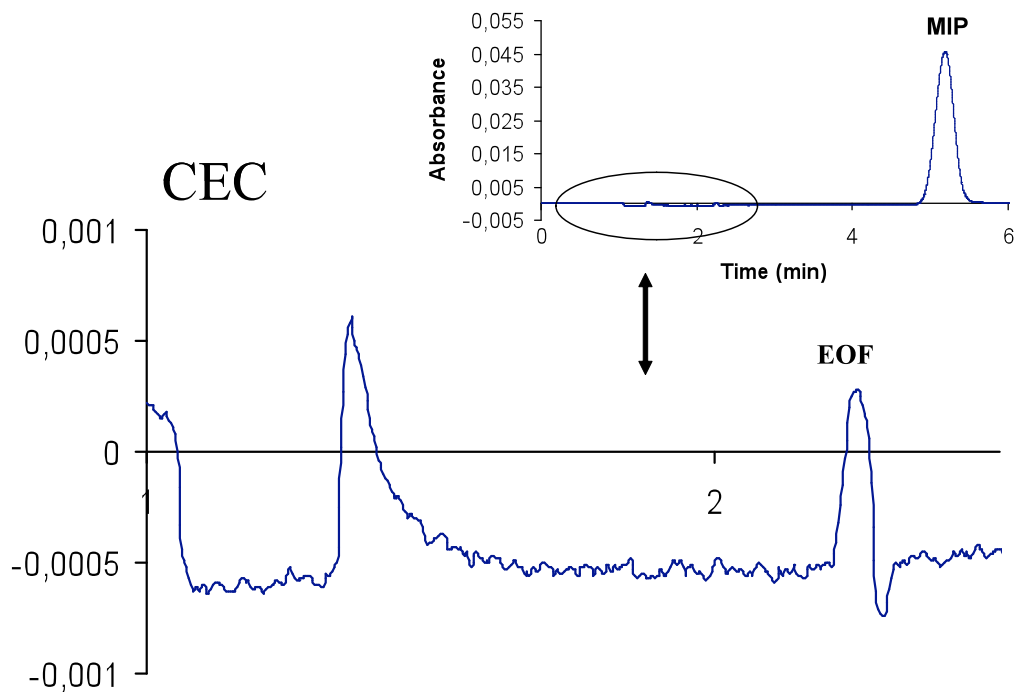
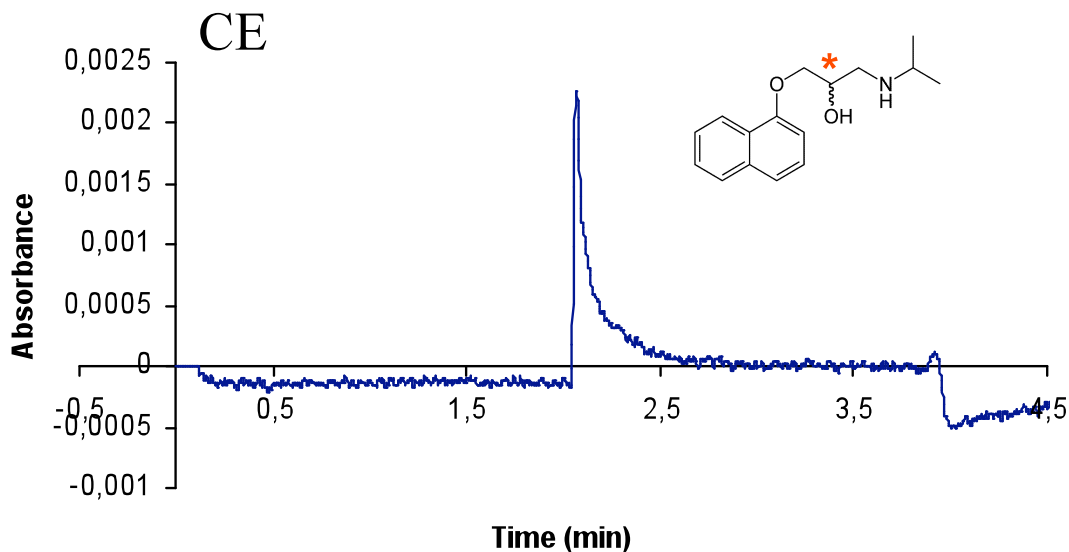


Figure 18- CE/CEC runs of propranolol. (A) Electropherogram of rac-propranolol (50 μ M) without MIP nanoparticles. (B) Electrochromatogram of rac-propranolol with MIP nanoparticles. Electrolyte composed of 70% of acetonitrile 50 mM ammonium acetate buffer (pH 6). Injection of the sample was made electrokinetically at 3 kV 2,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 3,0 s. Separation performed at 10 kV, room temperature. UV detection was made at 214 nm.

(A)



(B)

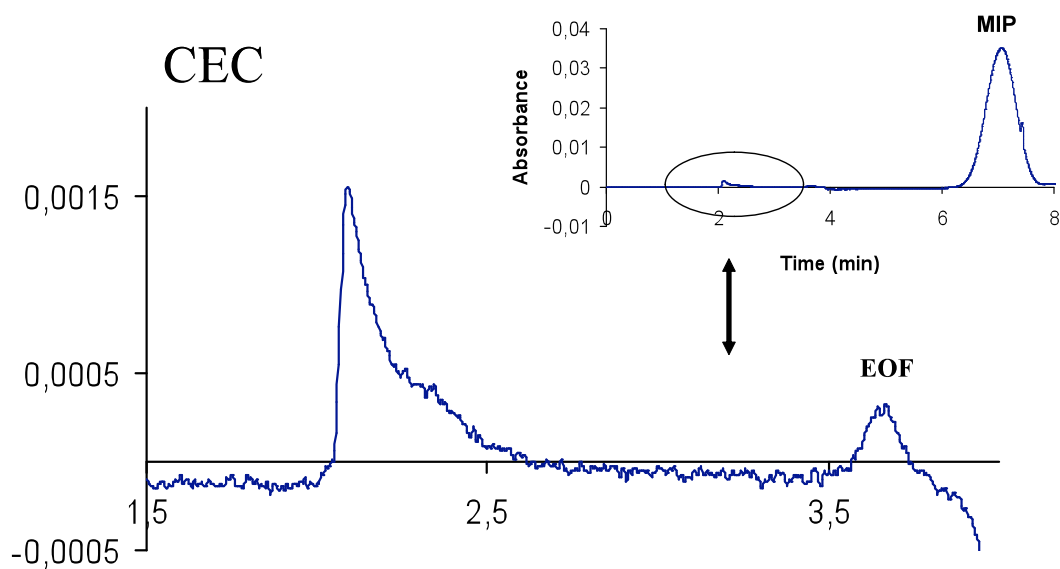
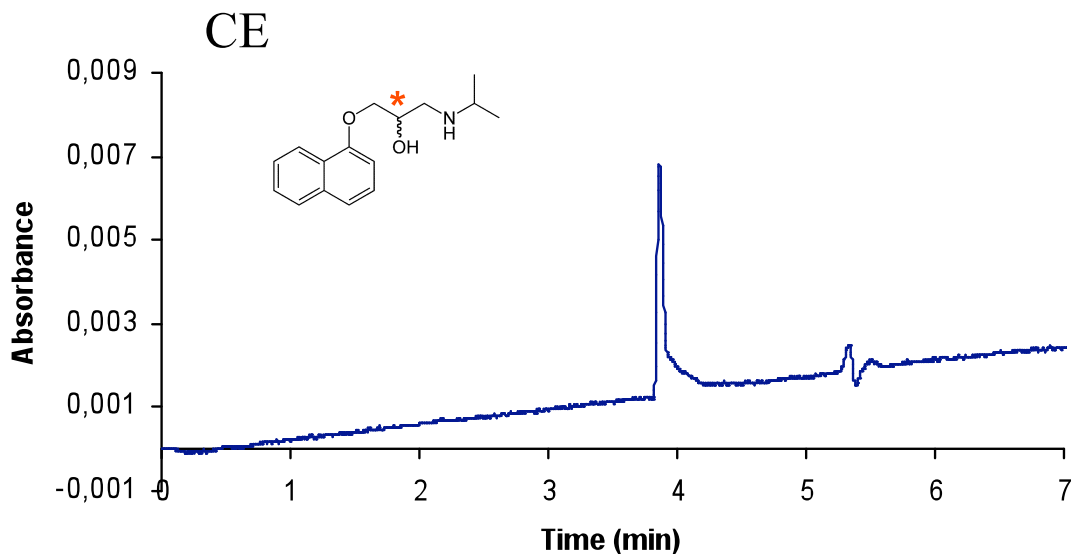


Figure 19- CE/CEC runs of propranolol. (A) Electropherogram of rac-propranolol ($50 \mu\text{M}$) without MIP nanoparticles. (B) Electrochromatogram of rac-propranolol with MIP nanoparticles. Electrolyte composed of 90% of acetonitrile 20 mM citrate buffer (pH 3). Injection of the sample was made electrokinetically at 3 kV 3,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 3,0 s. Separation performed at 3 kV, room temperature. UV detection was made at 214 nm.

(A)



(B)

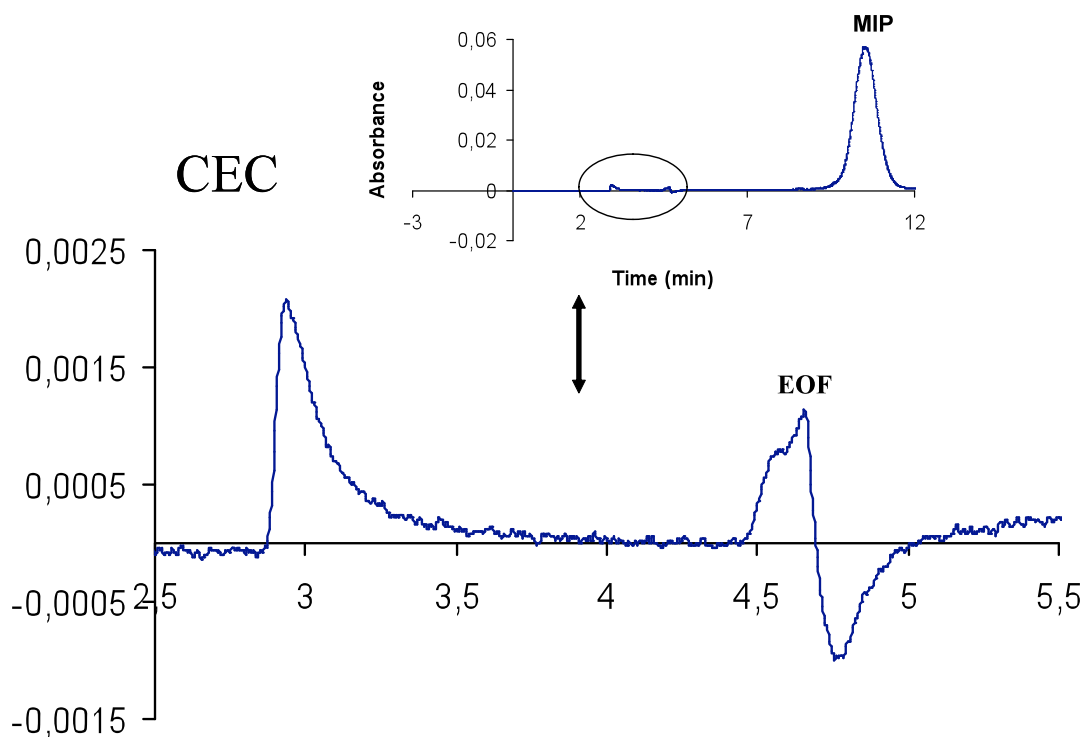


Figure 20- CE/CEC runs of propranolol. (A) Electropherogram of rac-propranolol (100 μ M) without nanoparticles. (B) Electrochromatogram of rac-propranolol with MIP nanoparticles. Electrolyte composed of 90% of acetonitrile 20 mM MES buffer (pH 7). Injection of the sample made electrokinetically at 3 kV 5,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 2,0 s. Separation performed at 3 kV, room temperature. UV detection was made at 214 nm.

3.1.2 Core shell MIP nanoparticles

Core shell MIP nanoparticles composed of a highly crosslinked MAA imprinted core with a shell of crosslinked poly(acrylamide) were synthesized (Figure 21). This non-imprinted shell makes the MIP nanoparticles more hydrophilic but the imprinting cavity presents hydrophobic characteristics due to the monomers. The nanoparticles were imprinted using R-propranolol as a template.

Average hydrodynamic size was measured by DLS. The MIP core of the nanoparticles has a radius of 82 nm and the MIP shell 130 nm.

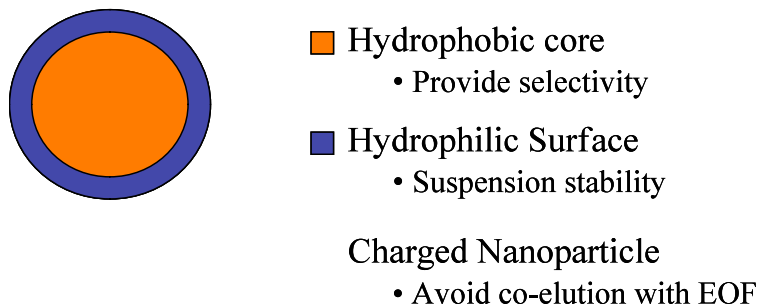


Figure 21-Nanoparticle schematic.

3.1.2.1 Suspension stability of nanoparticles in different electrolyte composition

The suspension stability of MIP nanoparticles in the electrolyte is important to achieve good performance in CEC. Different electrolyte conditions were tested in eppendorf tubes changing type of buffer, ionic strength, pH as well as the percentage of acetonitrile used (Table 7). The MIP nanoparticles were suspended in the electrolyte to a concentration of 5mg mL⁻¹.

Table 7- Composition of electrolyte tested. Nanoparticles sedimentated ☒, Stable√.

ACN Content (%v/v)	Citrate				MES		Phosphate	
	pH=3,4		pH=5,9		pH=6,5		pH=7,00	
	20mM	50mM	20mM	50mM	20mM	50mM	20mM	50mM
90	☒	☒	√	√	√	☒	√	√
70	☒	☒	☒	☒	√	√	☒	☒
50	√	☒	√	√	√	√	√	√

MIP nanoparticles show suspension stability at lower content of ACN as expected. Stable suspensions of MIP nanoparticles are obtained using MES buffer at low concentration, so further studies were made using this buffer with different ionic strength, pH and different amounts of acetonitrile.

Other electrolyte compositions with different buffers were also tested in CEC studies but did not result in enantiomer separation of propranolol.

3.1.2.2 CEC enantiomer separation of rac-propranolol

3.1.2.2.1 Enantiomer separation with MES buffer pH 6,5

Compositions of electrolytes using MES buffer pH 6,5 with different concentrations and various contents of ACN were tested in CEC (Table 8).

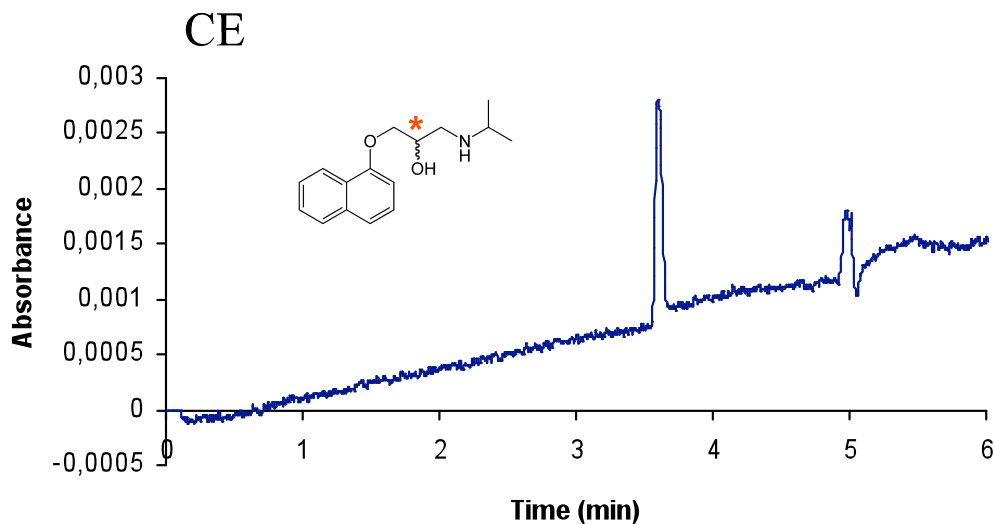
Table 8- *Composition of electrolytes used in CEC performance.*

Electrolyte solution	Acetonitrile Content (%v/v)
10mM MES	40
20mM MES	30
	40
	50
	60
	70
30mM MES	50
50mM MES	50

Enantiomer separation of propranolol was achieved using an electrolyte composition of 40% of acetonitrile and 60% 20mM MES buffer pH 6.5 (Figure 22). The MIP electrolyte was introduced hydrodynamically into the capillary during 10,0s at 0,5 psi to partially fill the capillary with MIP nanoparticles. The sample was then injected electrokinetically during 3,0s at 3,0 kV.

Enantiomers were separated in less than 4 min with the expected elution order of R-propranolol being the most retained.

(A)



(B)

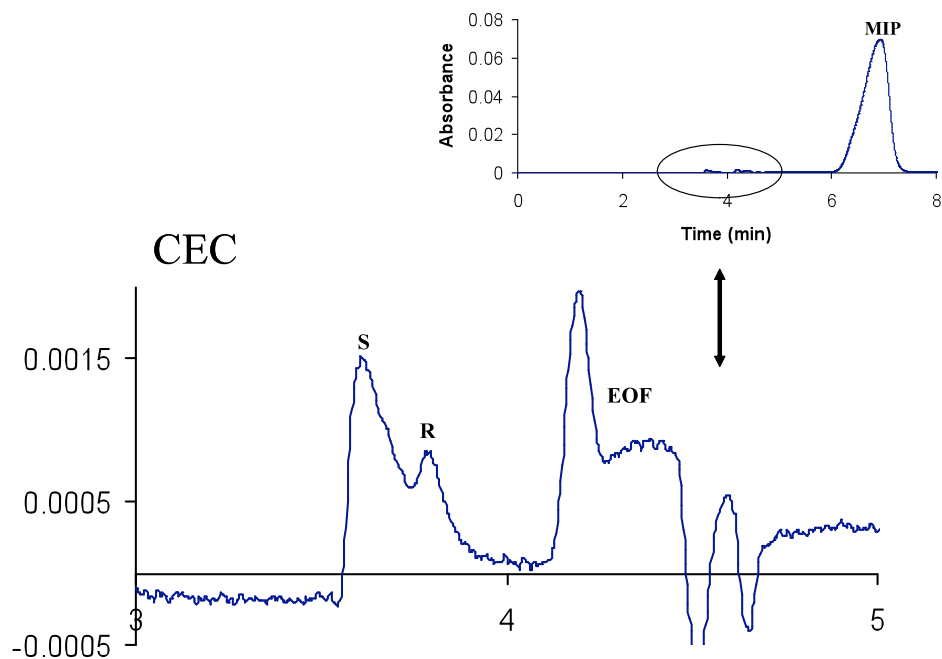


Figure 22- CE/CEC runs of propranolol. a) Electropherogram of rac-propranolol (50 μ M) without nanoparticles. b) Electrochromatogram of rac-propranolol with MIP nanoparticles. Electrolyte composed of 40% of acetonitrile and 60% 20 mM MES buffer (pH 6,5). Injection of the sample made electrokinetically at 3 kV 5,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 10,0 s. Separation performed at 3 kV, room temperature. UV detection was made at 214 nm.

Propranolol enantiomer separation by MIP nanoparticles results from specific interactions with recognition sites within the MIP nanoparticles but also nonspecific interactions with randomly hydrogen bonds residues can occur. The quantity of these nonspecific interactions can contribute to a higher or lower specificity.

Enantiomer separation of propranolol was possible but with time only one peak could be noticed (Figure 23, 24). The nonspecific interactions appear to have a strong influence on the separation. Tailing of the second peak are shown in figure 22 as a result of these unspecific interactions.

Strong interactions between the MIP nanoparticles and the capillary wall can occur even though flushing with NaOH is performed between each run.

Also evaporation of the organic solvent can become significant with time due to the design of vials used in this CEC instrument.

At higher concentrations, buffer ions can act as competing ligands and influence recognition. In this case, the influence of buffer molecules can be assumed to be negligible since low concentration was used.

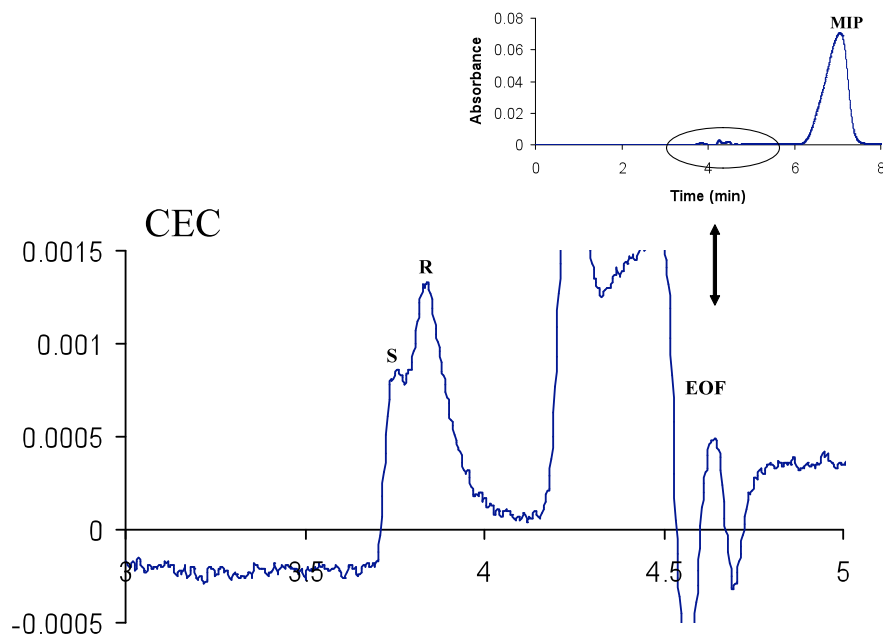


Figure 23- Electrochromatogram of rac-propranolol with MIP nanoparticles. Electrolyte composed of 40% of acetonitrile and 60% 20 mM MES buffer (pH 6,5). Injection of the sample made electrokinetically at 3 kV 5,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 10,0 s. Separation performed at 3 kV, room temperature. UV detection was made at 214 nm.

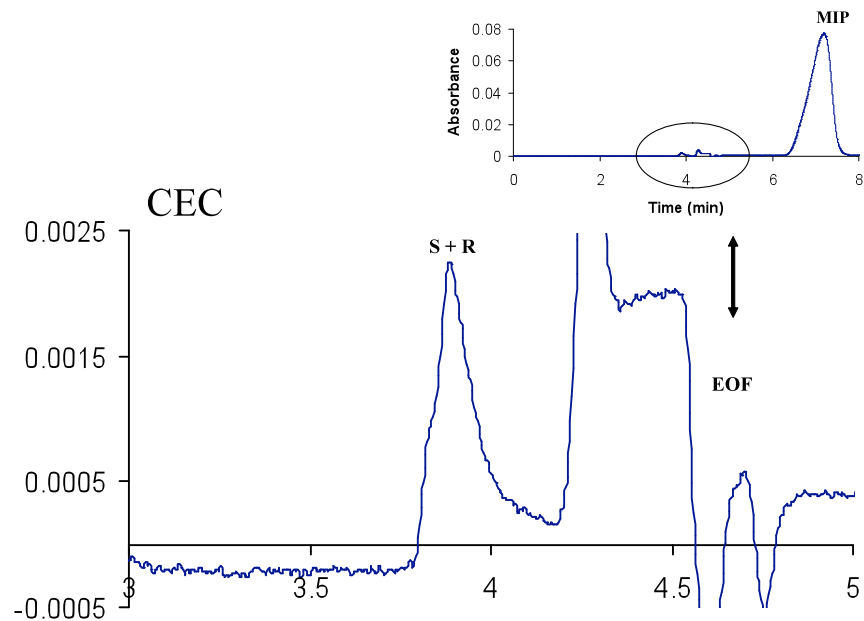


Figure 24- Electrochromatogram of *rac*-propranolol with MIP nanoparticles. Electrolyte composed of 40% of acetonitrile and 60% 20 mM MES buffer (pH 6,5). Injection of the sample made electrokinetically at 3 kV 5,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 10,0 s. Separation performed at 3 kV, room temperature. UV detection was made at 214nm.

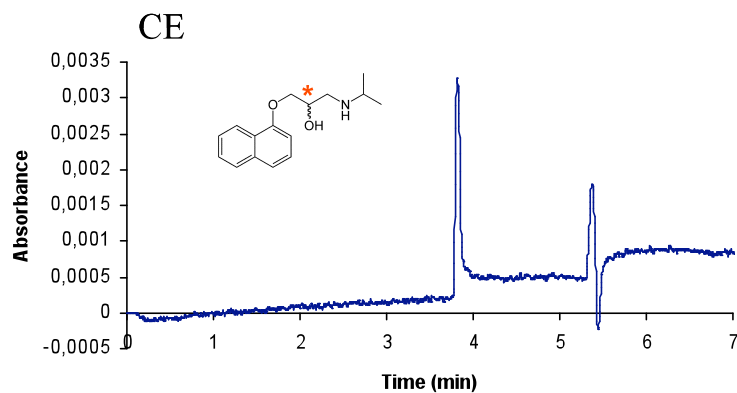
The experiments were performed with low levels of ACN to promote imprinting recognition based on hydrophobic effect. Addition of buffer with high pH to the electrolyte decreases the tendency of propranolol ($pK_a=9,4$) to be ionized and increase its hydrophobicity.

To increase the recognition based on hydrophobic interactions and decrease the influence of electrostatic interactions, a higher pH was used in the following studies.

3.2.2.2 Enantiomer separation with MES buffer pH=7

Enantiomer separation was possible at pH 7 with 40% ACN and 60% 20 mM MES as shown in figure 25. MIP nanoparticles were introduced hydrodynamically during 8s at 0,5psi and the sample injected electrokinetically for 5,0 s at 3,0 kV.

(A)



(B)

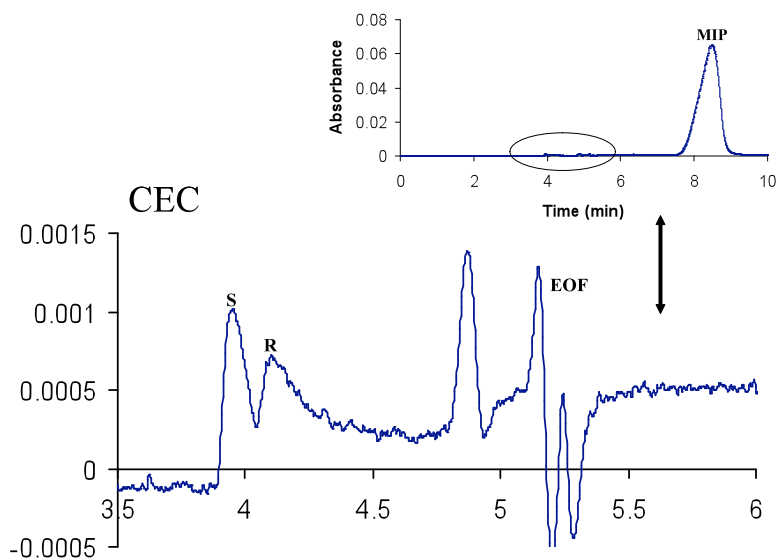


Figure 25- CE/CEC runs of propranolol. (A) Electropherogram of rac-propranolol (100 μ M) without nanoparticles. (B) Electrochromatogram of rac-propranolol with MIP nanoparticles. Electrolyte composed of 40% of acetonitrile and 60% 20 mM MES buffer (pH 7). Injection of the sample made electrokinetically at 3 kV 5,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 8,0 s. Separation performed at 3 kV, room temperature. UV detection was made at 214 nm.

Improvement of baseline separation was achieved in comparison with lower pH. But with time the behavior was the same as for pH 6,5 when only one peak could be seen (Figure 26, 27).

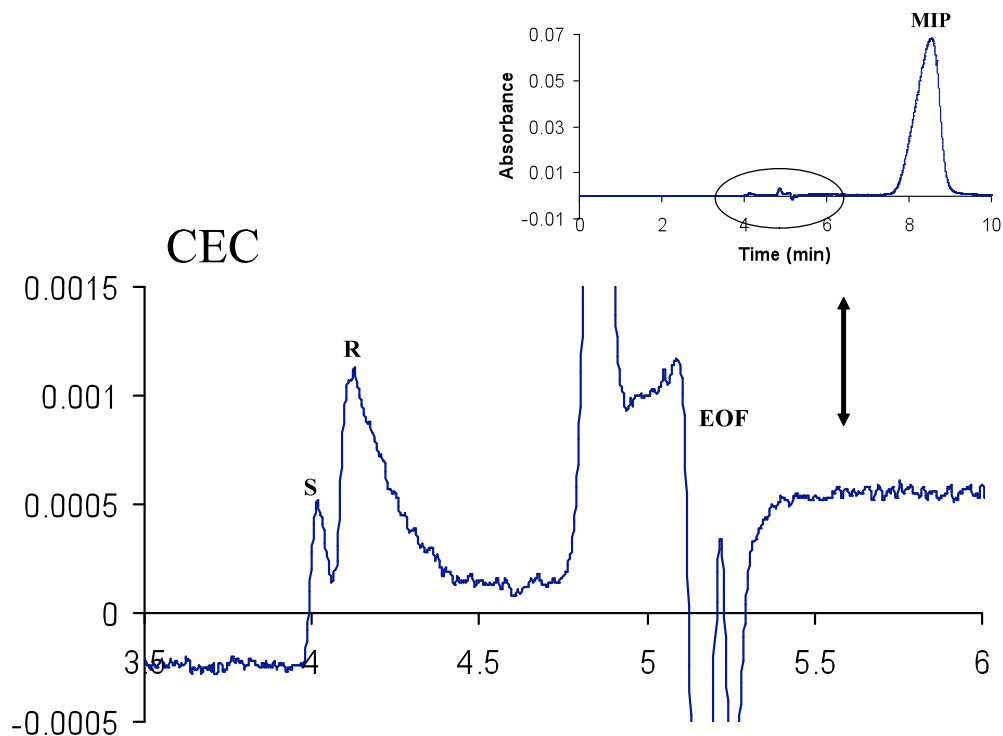


Figure 26- Electrochromatogram of rac-propranolol (100 μ M) with MIP nanoparticles. Electrolyte composed of 40% of acetonitrile and 60% 20 mM MES buffer (pH 7). Injection of the sample made electrokinetically at 3 kV 5,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 8,0 s. Separation performed at 3 kV, room temperature. UV detection was made at 214 nm.

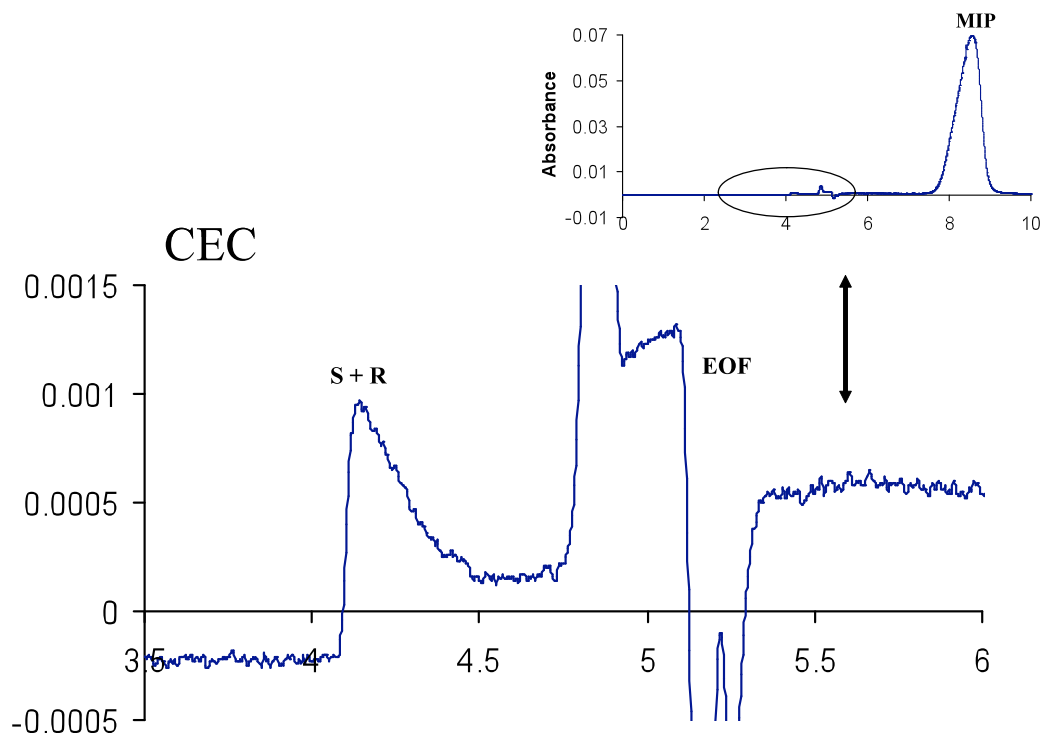


Figure 27- Electrochromatogram of *rac*-propranolol (100 μ M) with MIP nanoparticles. Electrolyte composed of 40% of acetonitrile and 60% 20mM MES buffer (pH 7). Injection of the sample made electrokinetically at 3 kV 5,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 8,0 s. Separation performed at 3 kV, room temperature. UV detection was made at 214 nm.

Even though hydrophobic interactions were favored it seems that unspecific interactions are still occurring. As explained before other factors can contribute for lower recognition, interaction between capillary wall and MIP nanoparticles and evaporation of the organic solvent.

To verify how strong the effect of unspecific interactions were, enantiomer separation samples of R (+) and S (-) of propranolol were injected separately.

As seen in figure 28 the R form interacts more strongly than the S form. This shows that the MIP nanoparticles have more specificity for the R form as expected.

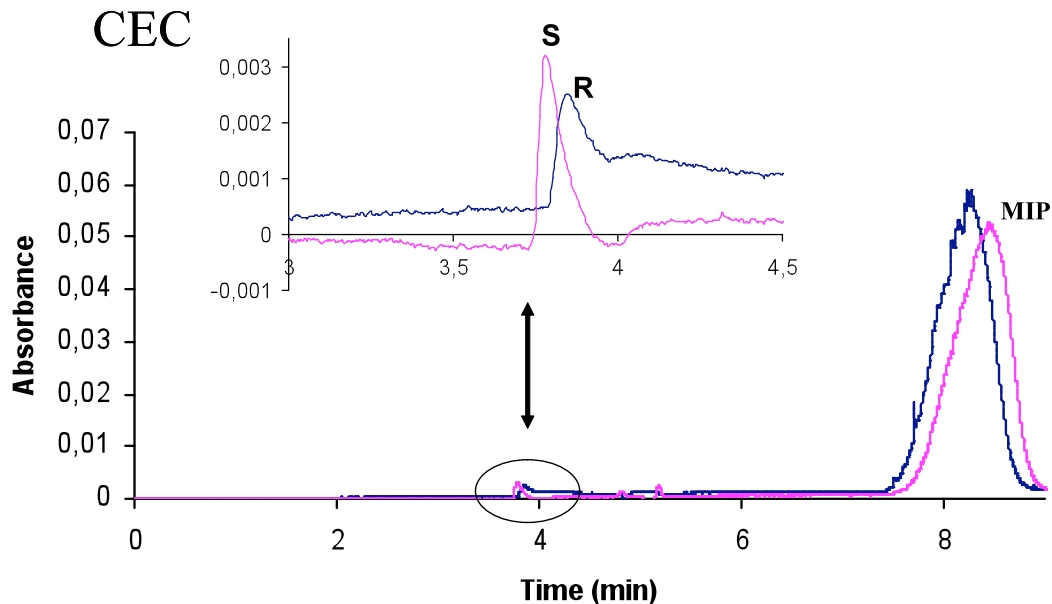


Figure 28- Electrochromatogram of (R)-propranolol 100 μ M (blue) and (S)-propranolol 100 μ M (pink) with MIP nanoparticles. Electrolyte composed of 40% of acetonitrile and 60% 20mM MES buffer (pH 7). Injection of the samples made electrokinetically at 3 kV 5,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 8,0s. Separation performed at 3 kV, room temperature. UV detection was made at 214 nm.

In order to increase the time of interaction between the MIP nanoparticles and the sample Tween 20, a non-ionic surfactant, was used as an electrolyte modifier in order to suppress the EOF. Previous studies show that the use of organic modifiers and/or surfactants can improve the performance of MIP-based CEC systems. In this study improvement was not noticed and enantiomers co-eluted (Figure 29). Also severe tailing of the peak was noticed.

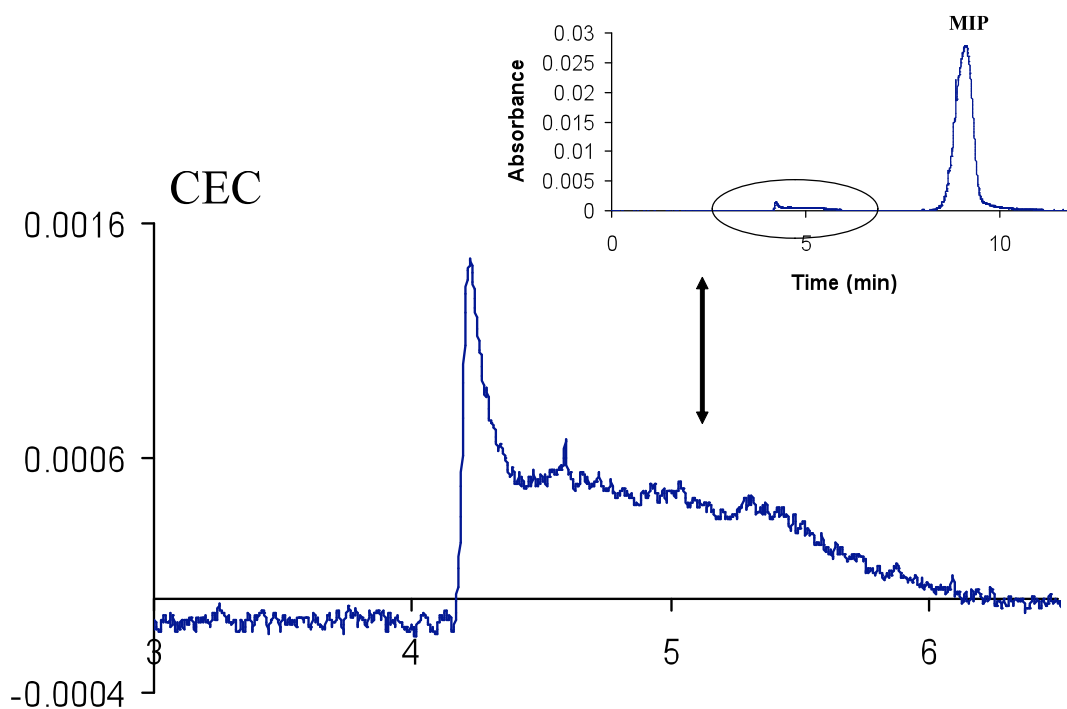


Figure 29- Electrochromatogram of rac-propranolol (100 μM) with MIP nanoparticles. Electrolyte composed of 0,05% Tween 20, 40% of acetonitrile and 59,95% 20 mM MES buffer (pH 7). Injection of the sample made electrokinetically at 3 kV 3,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 4,0 s. Separation performed at 3 kV, room temperature. UV detection was made at 214 nm.

Since enantiomer separation was almost successful with 40% ACN, small variations of ACN content were made in the electrolyte in order to get more reproducible results. Electrolytes compositions with 37% and 45% contents of ACN were used.

Table 9- Composition of electrolytes used in CEC performance.

Electrolyte solution	Acetonitrile Content (%v/v)
20mM MES	37
	45

In both situations enantiomers were co-eluted (Figure 30, 31).

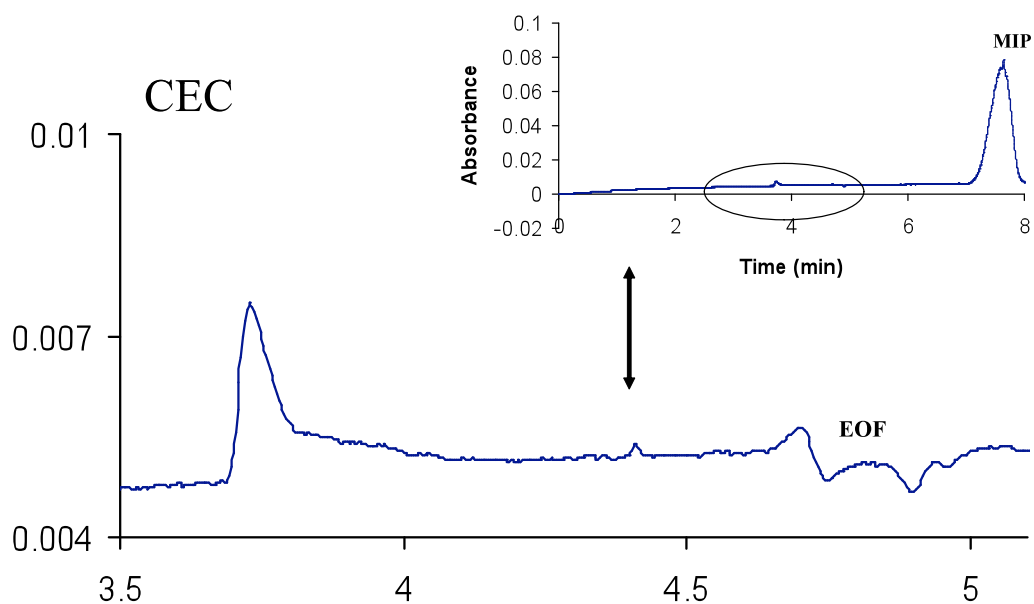


Figure 30-Electrochromatogram of rac-propranolol (100 μ M) with MIP nanoparticles. Electrolyte composed of 37% of acetonitrile and 63% 20 mM MES buffer (pH 7). Injection of the sample made electrokinetically at 3 kV 3,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 6,0 s. Separation performed at 3 kV, room temperature. UV detection was made at 214 nm.

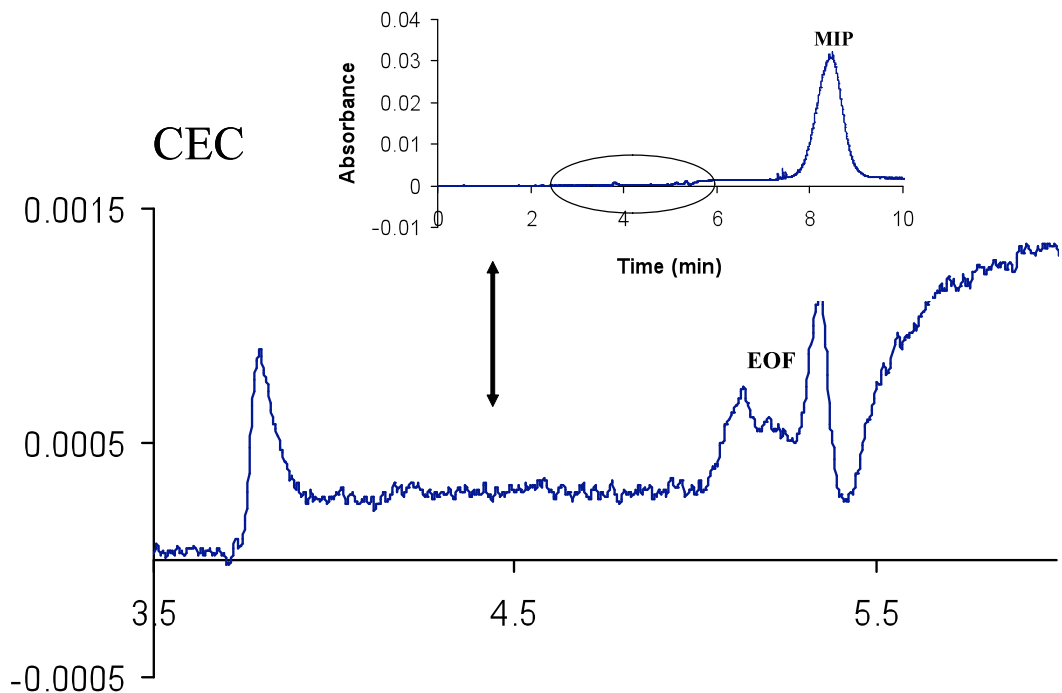


Figure 31- Electrochromatogram of rac-propranolol (100 μM) with MIP nanoparticles. Electrolyte composed of 45% of acetonitrile and 55% 20 mM MES buffer (pH 7). Injection of the sample made electrokinetically at 3 kV 3,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 6,0s. Separation performed at 3 kV, room temperature. UV detection was made at 214 nm.

To increase even more the influence of hydrophobic interactions less content of ACN was tested (Table 10).

Table 10- Composition of electrolytes used in CEC performance.

Electrolyte solution	Acetonitrile Content (%v/v)
20mM MES	20
	30

As seen in Figure 32, 33 enantiomer separations were not achieved and enantiomers co-eluted. Tailing of the peaks can be noticed due to the unspecific interactions.

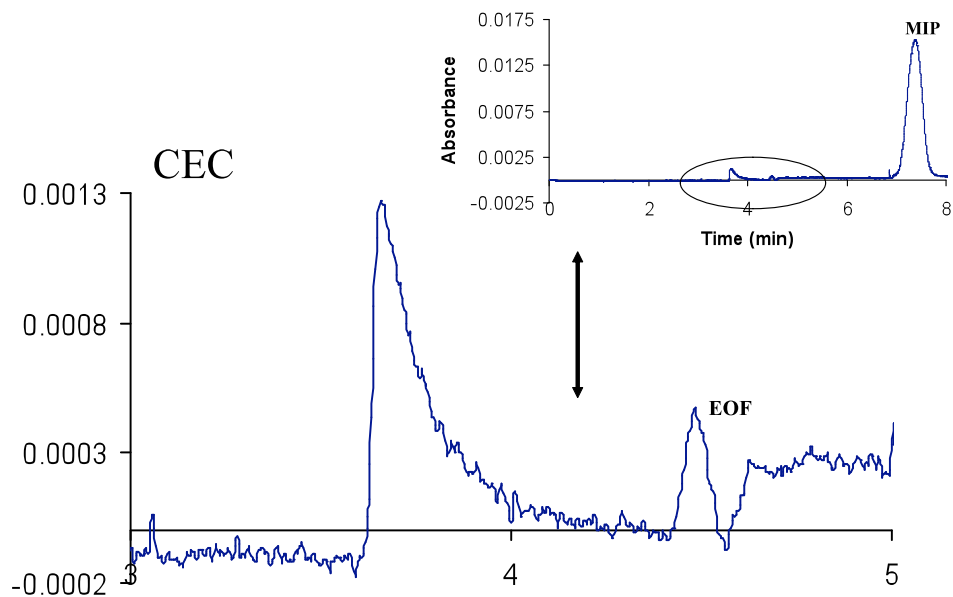


Figure 32-Electrochromatogram of rac-propranolol (100 μ M) with MIP nanoparticles. Electrolyte composed of 20% of acetonitrile and 80% 20 mM MES buffer (pH 7). Injection of the sample made electrokinetically at 3 kV 5,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 4,0 s. Separation performed at 3 kV, room temperature. UV detection was made at 214 nm.

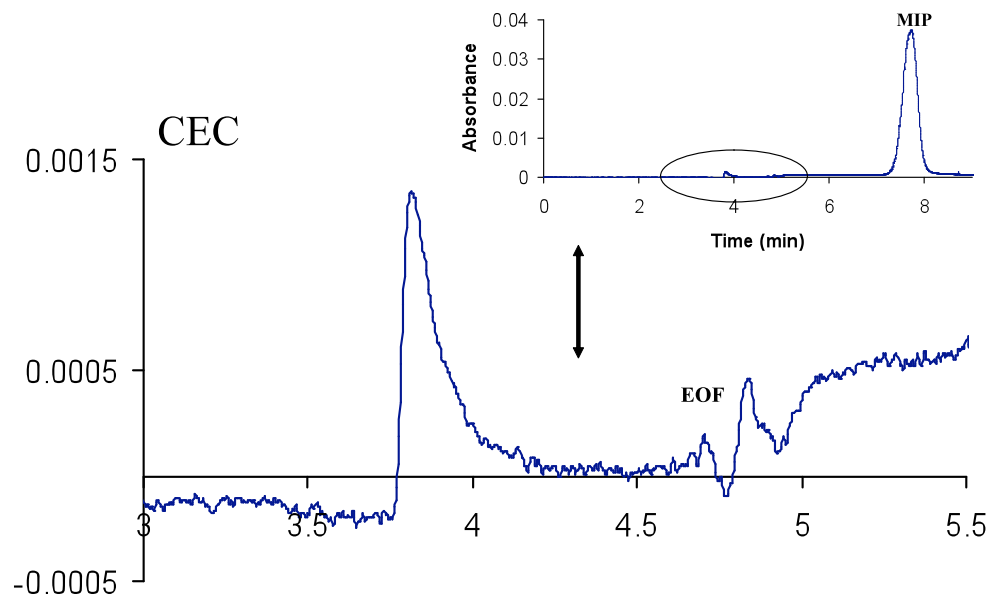


Figure 33- Electrochromatogram of rac-propranolol (100 μ M) with MIP nanoparticles. Electrolyte composed of 30% of acetonitrile and 70% 20 mM MES buffer (pH 7). Injection of the sample made electrokinetically at 3 kV 6,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 2,0s. Separation performed at 3 kV, room temperature. UV detection was made at 214 nm.

Also experiments with high percentage of ACN were performed. In this case electrostatic interactions will be favored. In Table 11 the composition of electrolytes used are summarized.

Table 11- Composition of electrolytes used in CEC performance.

Electrolyte solution	Acetonitrile Content (%v/v)
20mM MES	50
	70

As observed in figures 34, 35 the increasing of ACN resulted in co-elution of enantiomers.

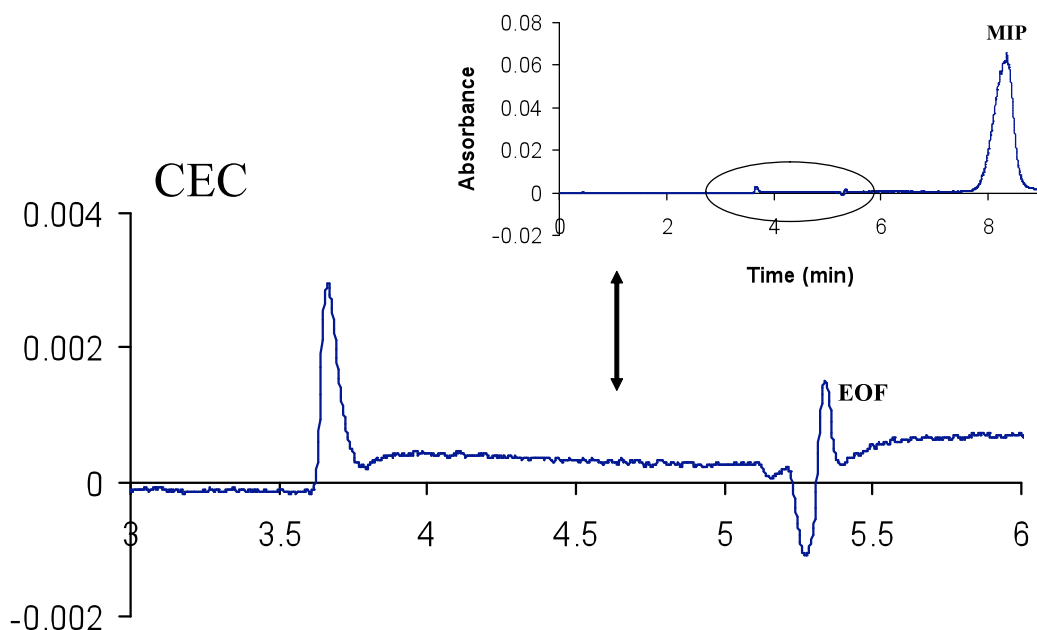


Figure 34- Electrochromatogram of rac-propranolol (100 μ M) with MIP nanoparticles. Electrolyte composed of 50% of acetonitrile and 50% 20mM MES buffer (pH 7). Injection of the sample made electrokinetically at 3 kV 3,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 8,0 s. Separation performed at 3 kV, room temperature. UV detection was made at 214 nm.

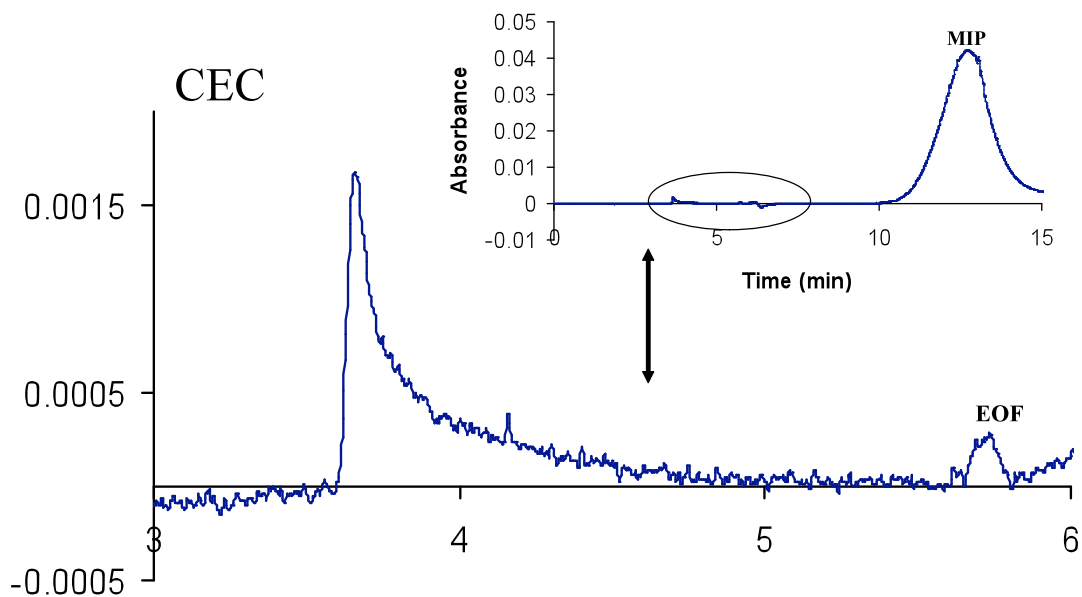


Figure 35- Electrochromatogram of rac-propranolol (100 μ M) with MIP nanoparticles. Electrolyte composed of 70% of acetonitrile and 30% 20 mM MES buffer (pH 7). Injection of the sample made electrokinetically at 3 kV 3,0 s; MIP nanoparticles injection made hydrodynamically at 0,5 psi 8,0 s. Separation performed at 3 kV, room temperature. UV detection was made at 214 nm.

3.2 Silanized gadolinium oxide nanoparticles tested for protein separation

Protein separation in CE can be a problem due to the adsorption of biomolecules to the negatively charged surface of the capillary wall. Electrostatic and hydrophobic interactions as well as structural changes in the protein can be the main reasons for the interactions and attachment of the proteins to the surface of the capillary. Electrostatic interactions depend on the surface charge and the protein charge, both of which are dependent on pH and electrolyte composition. Hydrophobic interactions results from the binding of hydrophobic moieties of the protein to hydrophobic surfaces.

Normally to analyze proteins in CEC coupling with either MS or LIF is necessary for protein detection.

A new strategy to optimize protein separation is presented in this work with the use of silanized gadolinium oxide nanoparticles as a pseudostationary phase in CEC.

The average size of these nanoparticles is 4-5nm without silica coating and 15nm with the coating. At physiological pH this nanoparticles are negatively charged due to the silica groups. Due to the well know chemistry of silica, derivatization of these groups is possible allowing attachment of different ligands.

A hydrodynamic injection of nanoparticles alone was done during 10s at 0,5psi. As seen in Figures 36, 37 the nanoparticles could not be detected. Due to the lack of disturbance in detection and to the large surface area, these nanoparticles show promising application in protein separation as a pseudostationary phase in CEC combined with UV detection.

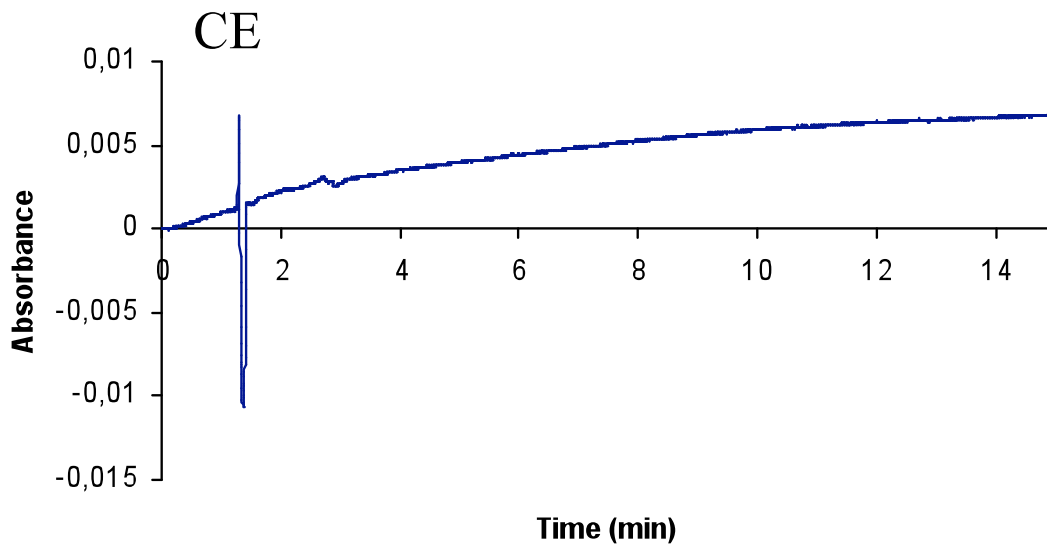


Figure 36- Electropherogram of silica-coated gadolinium nanoparticles. Electrolyte composed of 50 mM Tricine buffer pH 7,5. Injection of the nanoparticles was made hydrodynamically during 10 s at 0,5 psi. Separation performed at 10 kV, room temperature. UV detection was made at 200nm.

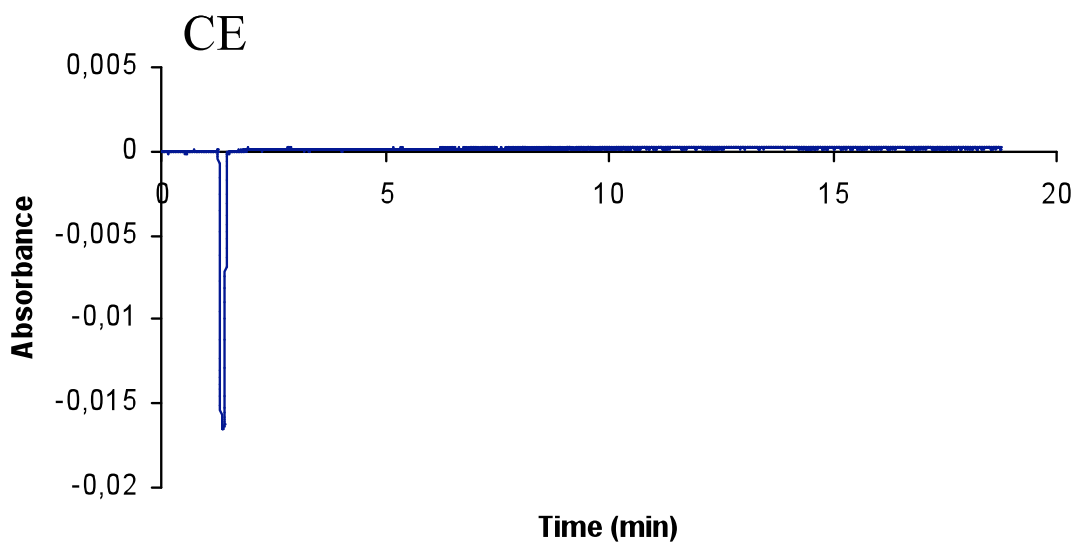


Figure 37- Electropherogram of silica-coated gadolinium nanoparticles. Electrolyte composed of 50 mM Tricine buffer pH 7,5. Injection of the nanoparticles was made hydrodynamically during 10 s at 0,5 psi. Separation performed at 10 kV, room temperature. UV detection was made at 254nm.

Partial filling technique was used to analyze two types of proteins using silanized gadolinium oxide nanoparticles, lysozyme (pI=10,7) and human growth hormone (hGH, pI=5,2). The experiments were performed at physiological pH where lysozyme is positively charged and hGH negatively charged (Figure 38).

The hGH was injected electrokinetically during 3,0s at 3,0kV. The nanoparticle electrolyte was introduced hydrodynamically during different times at 0,5psi. As seen in Figure 39 increasing times of nanoparticle injection gives smaller, broader and more retained peaks of the hGH, revealing that interactions between the sample and nanoparticles are occurring.

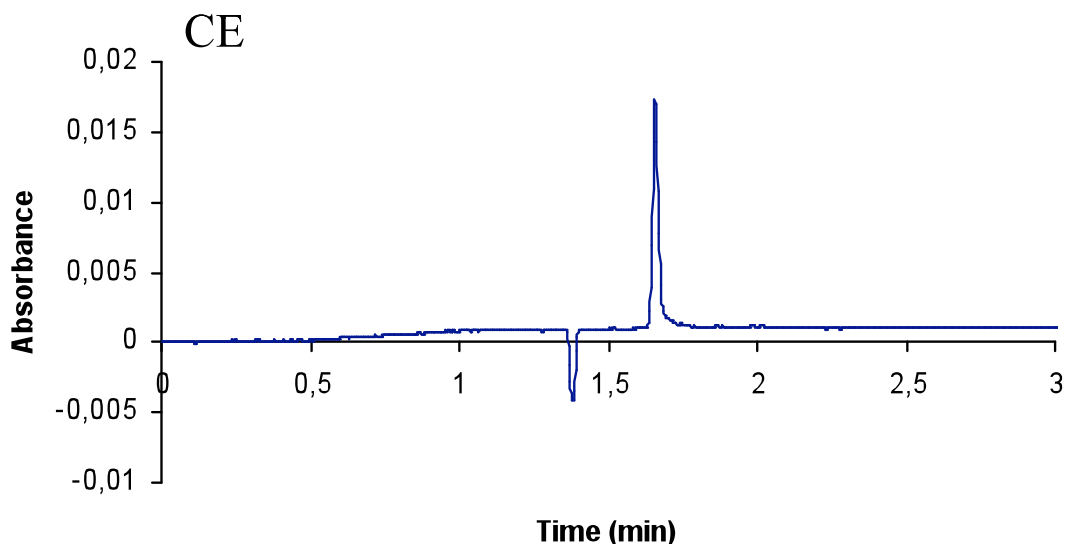


Figure 38- Electropherogram of hGH (0,5mg/mL) without nanoparticles. Electrolyte composed of 100mM Tricine buffer pH 7,5. Injection of the sample was made electrokinetically during 3,0s at 3,0kV. Separation performed at 10kV, room temperature. UV detection was made at 200 nm.

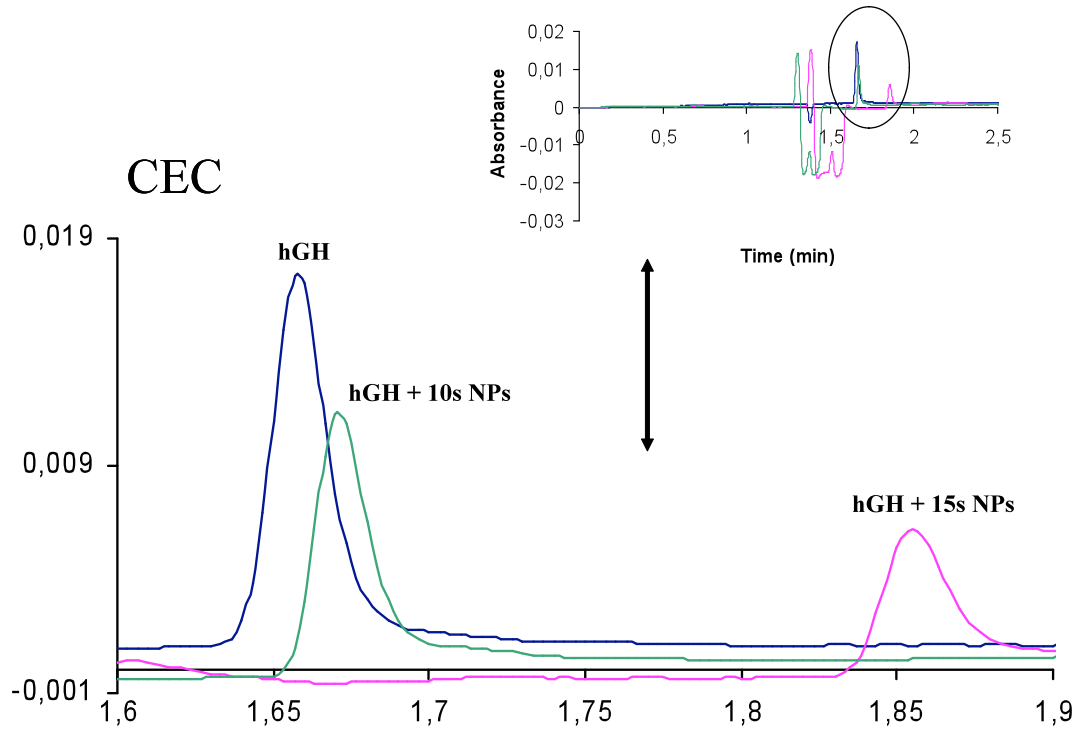


Figure 39- Electrochromatogram of hGH with nanoparticles. Electrolyte composed of 100 mM Tricine buffer pH 7,5. Injection of the sample was made electrokinetically during 3,0 s at 3,0 kV. Nanoparticle injection was made hydrodynamically during 10s (green) and 15s (pink) at 0,5 psi. Separation performed at 10 kV, room temperature. UV detection was made at 200 nm.

Lysozyme at physiological pH is positively charge and can not be detected in CE (Figure 40) due to the adsorption of the protein molecules to the negative charged surface of the fused-silica capillary.

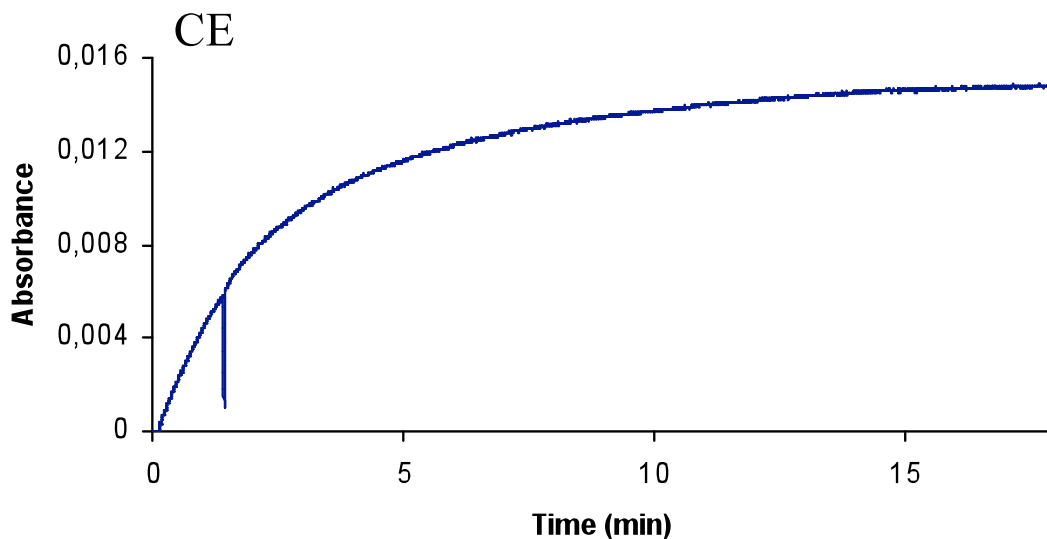


Figure 40- Electropherogram of lysozyme (0,5mg/mL) without nanoparticles. Electrolyte composed of 100 mM Tricine buffer pH 7,5. Injection of the sample was made electrokinetically during 3,0 s at 3,0 kV. Separation performed at 10kV, room temperature. UV detection was made at 200 nm.

By adding nanoparticles to the electrolyte it was possible to see a peak (Figure 41, 42). One possibility is that he nanoparticles act as a coating in the capillary and reduce the interactions between the protein and the capillary. Another reason can be the large surface area of the nanoparticles which can obstruct the adsorption of the protein to the capillary wall.

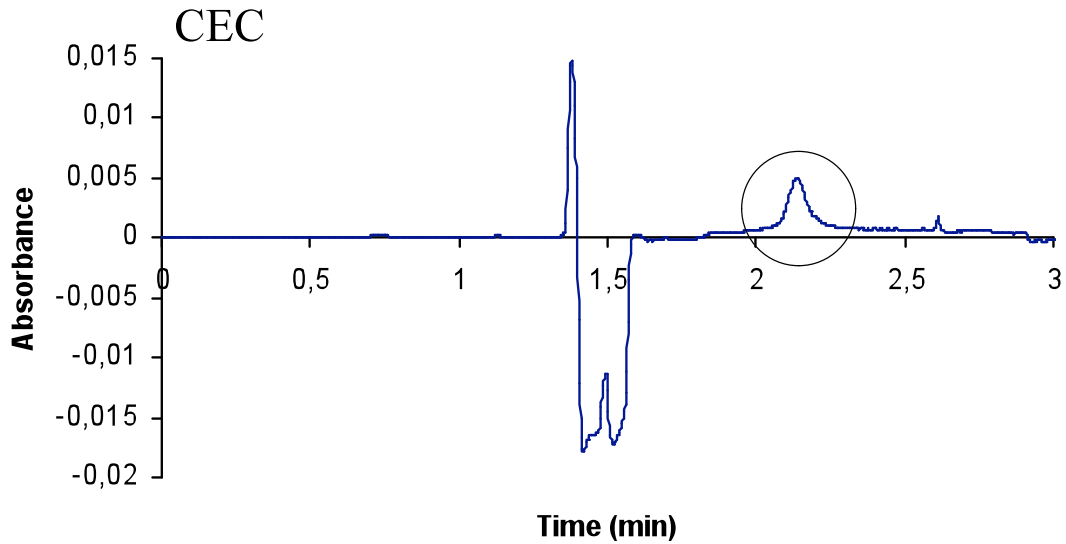


Figure 41- Electrochromatogram of lysozyme with nanoparticles. Electrolyte composed of 100 mM Tricine buffer pH 7,5. Injection of the sample was made electrokinetically during 3,0 s at 3,0 kV. Nanoparticle injection was made hydrodynamically during 15 s at 0,5 psi. Separation performed at 10 kV, room temperature. UV detection was made at 200 nm.

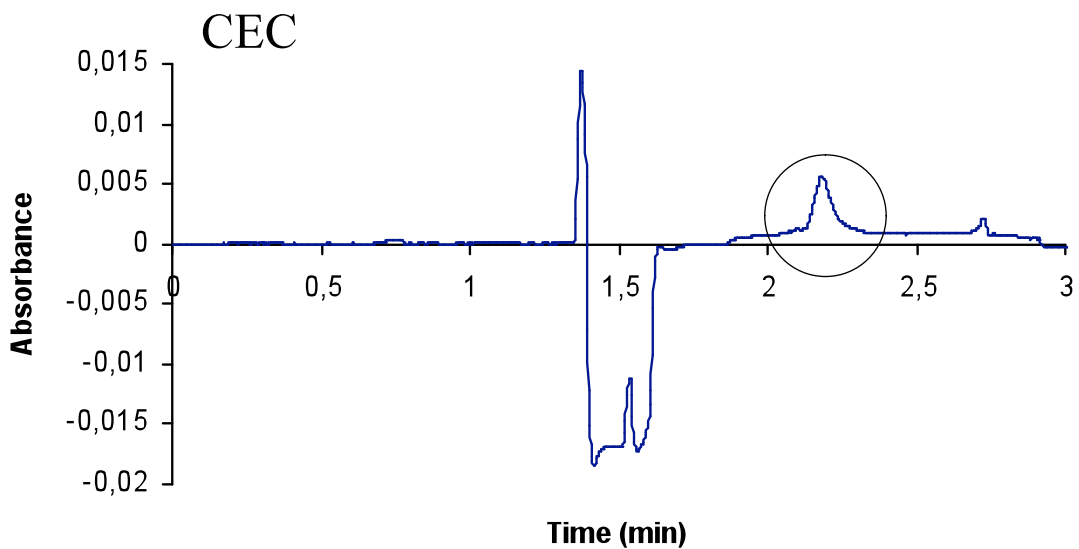


Figure 42- Electrochromatogram of lysozyme with nanoparticles. Electrolyte composed of 100 mM Tricine buffer pH 7,5. Injection of the sample was made electrokinetically during 3,0 s at 3,0 kV. Nanoparticle injection was made hydrodynamically during 20 s at 0,5 psi. Separation performed at 10 kV, room temperature. UV detection was made at 200 nm.

Strong electrostatic interactions between the nanoparticles and lysozyme occur making the lysozyme peak coming after the EOF. Since the lysozyme and the nanoparticles present different charge, a complex can be formed between them making the protein elute with the nanoparticles.

To prove that the peak seen was from lysozyme, a sample with only water was used. As seen in figure 43 nothing was detected confirming the detection of lysozyme in the previous experiments.

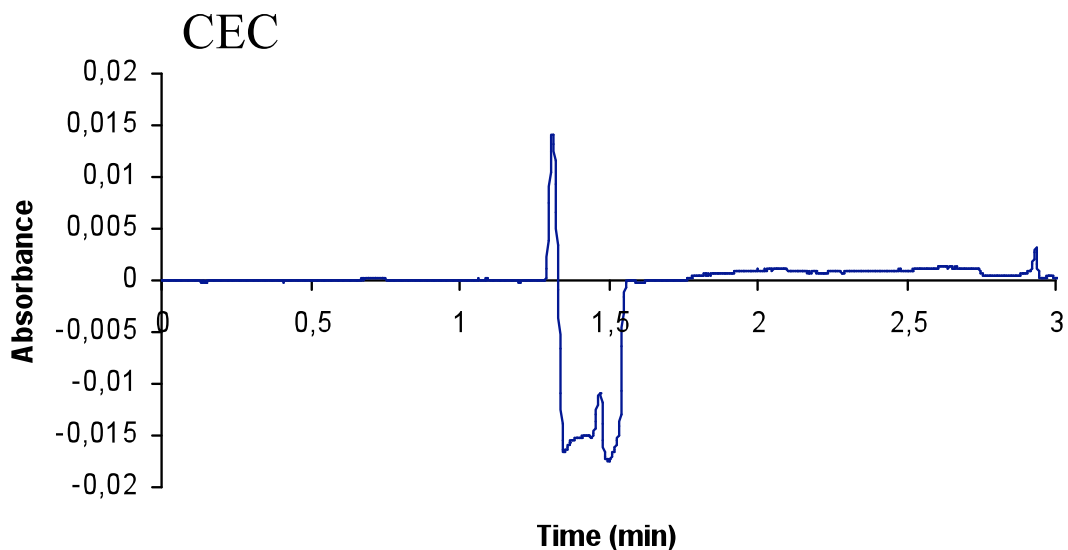


Figure 43- Electrochromatogram of water sample with nanoparticles. Electrolyte composed of 100 mM Tricine buffer pH 7,5. Injection of the sample was made electrokinetically during 3,0 s at 3,0 kV. Nanoparticle injection was made hydrodynamically during 20 s at 0,5 psi. Separation performed at 10 kV, room temperature. UV detection was made at 200 nm.

Silanized gadolinium oxide nanoparticles show a promising future in protein separation in CEC. The lack of disturbance in detection makes them suitable for this type of technique.

Further studies are necessary to improve the use of these nanoparticles as a pseudostationary phase. Continuous full filling technique would be next step to perform due to the characteristics of silanized gadolinium oxide nanoparticles.

4. Conclusion and future outlook

Enantiomers of propranolol were separated with reasonable efficiency using core-shell MIP nanoparticles. The structure of these nanoparticles with a hydrophobic core and a hydrophilic surface allows operation in more aqueous electrolytes favoring hydrophobic interactions between nanoparticles core and propranolol, which contributes to efficient separation of both enantiomers.

With methacrylic acid based nanoparticles enantiomer separation of propranolol was not achieved. Suspension stability of the nanoparticles was a major problem restricting the electrolytes compositions that can be used in MIP-CEC.

The potential of silanized gadolinium oxide nanoparticles as a pseudostationary phase for protein separation was investigated in CEC mode. The lack of detection and the large surface area of these nanoparticles make them a promising tool in CEC protein separation.

hGH was retained when injection times of the nanoparticles was increased showing that interactions between them occur.

It was possible to detect lysozyme with the use of these nanoparticles which avoid adsorption of the protein to the capillary wall.

Further research is necessary to more clearly understand the performance of these nanoparticles. The use of continuous full filling would be favorable with silanized gadolinium oxide nanoparticles due to their specific characteristics. The most important feature is that they don't interfere in UV detection. Also the silica groups presented in these nanoparticles will allow the manipulation of the surface due to the well known chemistry of silica.

5. References

- (1) Heiger, D. *High Performance Chromatography*, 2000.
- (2) Tagliaro, F.; Manetto, G.; Crivellente, F.; Smith, F. P. *Forensic Science International* **1998**, *92*, 75-88.
- (3) Poole, C. F. *The Essence of Chromatography*, 1th ed.; Elsevier, 2003.
- (4) D. Skoog, F. H., T. Nieman. *Principles of Instrumental Analysis*, 5th ed., 1998.
- (5) Karger, B. L. *Current Opinion in Biotechnology* **1992**, *3*, 59-64.
- (6) Pretorius, V.; Hopkins, B. J.; Schieke, J. D. *Journal of Chromatography* **1974**, *99*, 23-30.
- (7) Jorgenson, J. W.; Lukacs, K. D. *Journal of Chromatography* **1981**, *218*, 209-216.
- (8) Knox, J. H.; Grant, I. H. *Journal of Chromatographia* **1991**, *32*, 317-328.
- (9) Rathore, A. S.; Horvath, C. *Analytical Chemistry* **1998**, *70*, 3069-3077.
- (10) Eeltink, S.; Svec, F.; *Electrophoresis* **2007**, *28*, 137-147.
- (11) Colón, L.A.; Burgos, G.; Maloney, T.D.; José M. Cintrón; Ramón L. Rodríguez *Electrophoresis* **2000**, *21*, 3965-3993.
- (12) Frame, L. A.; Robinson, M. L.; Lough, W. J. *Journal of Chromatography A* **1998**, *798*, 243-249.
- (13) Svec, F.; *Journal of Separation Science* **2005**, *28*, 729-745.
- (14) Guihen, E.; Glennon, J. D. *Journal of Chromatography A* **2004**, *1044*, 67-81.
- (15) Nilsson, C.; S.Nilsson *Electrophoresis* **2006**, *27*, 76-83.
- (16) Tang, Q.; Lee, M. L. *TrAC Trends in Analytical Chemistry* **2000**, *19*, 648-663.
- (17) Lynen, F.; Buica, A.; Villiers, A.; Crouch, A.; Sandra, P.; *Journal of Separation Science* **2005**, *28*, 1539-1549.
- (18) Angus, P. D. A.; Demarest, C. W.; Catalano, T.; Stobaugh, J. F. *Journal of Chromatography A* **2000**, *887*, 347-365.
- (19) Carney, R. A.; Robson, M. M.; Bartle, K. D.; Meyers, P. **1999**, *22*, 29-32.
- (20) Rebscher, H.; Pyell, U. *Journal of Chromatographia* **1994**, *38*, 11-12.
- (21) Allen, D.; El Rassi, Z. *Electrophoresis* **2003**, *24*, 3962-3976.

- (22) Quaglia, M.; Sellergren, B.; De Lorenzi, E. *Journal of Chromatography A* **2004**, *1044*, 53-66.
- (23) Tsuda, T.; Nomura, K.; Nakagawa, G. *Journal of Chromatography A* **1982**, *248*, 241-247.
- (24) Jinno, K.; Sawada, H. *TrAC Trends in Analytical Chemistry* **2000**, *19*, 664-675.
- (25) Terabe, S.; Otsuka, K.; Ichikawa, K.; Tsuchiya, A.; Ando, T. *Analytical Chemistry* **1984**, *56*, 111-113.
- (26) Wallingford, R. A.; Ewing, A. G. *Adv. Chromatogr.* **1989**, *29*, 65-67.
- (27) Palmer, C. P.; *Electrophoresis* **2002**, *23*, 3993-4004.
- (28) Bächmann, K.; Göttlicher, B.; Haag, I.; Han, K.-Y.; Hensel, W.; Mainka, A. *Journal of Chromatography A* **1994**, *688*, 283-292.
- (29) Göttlicher, B.; Bächmann, K. *Journal of Chromatography A* **1997**, *780*, 63-73.
- (30) Bächmann, K.; Göttlicher, B. *Chromatographia* **1997**, *45*, 249-254.
- (31) Fujimoto, C.; Muranaka, Y. *Journal of High Resolution Chromatography* **1997**, *20*, 400-402.
- (32) Neiman, B.; Grushka, E.; Gun, J.; Lev, O. *Analytical Chemistry* **2002**, *74*, 3484-3491.
- (33) Huang, M.-F.; Kuo, Y.-C.; Huang, C.-C.; Chang, H.-T. *Analytical Chemistry* **2003**, *76*, 192-196.
- (34) Tseng, W. L.; Huang, M. F.; Huang, Y. F.; Chang, H. T. *Electrophoresis* **2005**, *26*, 3069-3075.
- (35) Palmer, C. P.; Hancock, K.; McNair, H.; Tyndall, W.; Morris, S., Riva del Garda, Italy 1993.
- (36) Breadmore, M. C.; Macka, M.; Haddad, P. R. *Electrophoresis* **1999**, *20*, 1987-1992.
- (37) Viberg, P.; Jornten-Karlsson, M.; Petersson, P.; Spegel, P.; Nilsson, S. *Analytical Chemistry* **2002**, *74*, 4595-4601.
- (38) Na, N.; Hu, Y.; Ouyang, J.; Baeyens, W. R. G.; Delanghe, J. R.; Beer, T. D. *Analytica Chimica Acta* **2004**, *527*, 139-147.
- (39) Kroto, H. W.; R.Heath, J.; O'Brien, S. C.; R.F.Curl; Smalley, R. E. *Nature* **1985**, *318*, 162.

- (40) Treubig, J. M.; Brown, P. R. *Journal of Chromatography A* **2000**, 873, 257-267.
- (41) Wang, Z.; Luo, G.; Chen, J.; Xiao, S.; Wang, Y. *Electrophoresis* **2003**, 24, 4181-4188.
- (42) Xiong, X.; Ouyang, J.; Baeyens W.R.G.; Delanghe, J.; Shen, X; Yang, Y. *Electrophoresis* **2006**, 27, 3243-3253.
- (43) Nilsson, C.; Birnbaum, S.; Nilsson, S. *Journal of Chromatography A Editors' Choice I* **2007**, 1168, 212-224.
- (44) Valtcheva, L.; Mohammad, J.; Pettersson, G.; Hjertén, S. *Journal of Chromatography A* **1993**, 638, 263-267.
- (45) Schweitz, L.; Spégel, P.; Nilsson, S. *Analyst* **2000**, 125, 1899-1901.
- (46) Göttlicher, B.; Bächmann, K. *Journal of Chromatography A* **1997**, 768, 320-324.
- (47) Amini, A.; Paulsen-Sörman, U.; Westerlund, D. *Chromatographia* **1999**, 50, 7-8.
- (48) Tanaka, Y.; Terabe, S. *Journal of Chromatography A International Symposium on Molecular Chirality* **1995**, 694, 277-284.
- (49) Wulff, G. *Angew. Chem. Int. Ed. Engl.* **1995**, 34, 1812-1832.
- (50) Mosbach, K.; Ramström, O. *Bio/Technology* **1996**, 14, 163-170.
- (51) Sellergren, B.; Allender, C. J. *Advanced Drug Delivery Reviews Molecularly Imprinted Polymers: Technology and Applications* **2005**, 57, 1733-1741.
- (52) Yoshimatsu, K.; Reimhult, K.; Krozer, A.; Mosbach, K.; Sode, K.; Ye, L. *Analytica Chimica Acta* **2007**, 584, 112-121.
- (53) Schweitz, L.; Spégel, P.; Nilsson, S. *Electrophoresis* **2001**, 22, 4053-4063.
- (54) Ye, L.; Cormack, P. A. G.; Mosbach, K. *Anal. Commun.* **1999**, 36, 35-38.
- (55) Spégel, P.; Schweitz, L.; Nilsson, S. *Electrophoresis* **2003**, 24, 3892-3899.
- (56) Wistuba, D.; Schurig, V. *Journal of Chromatography A* **2000**, 875, 255-276.
- (57) Lin, J.-M.; Uchiyama, K.; Hobo, T. *Chromatographia* **1998**, 47, 11-12.
- (58) Lin, J.-M.; Nakagama, T.; Uchiyama, K.; Hobo, T. *Journal of Liquid Chromatography & Related Technologies* **1997**, 20, 1489-1506.
- (59) Reimhult, K.; Yoshimatsu, K.; Risveden, K.; Chen, S.; Ye, L.; Krozer, A. *Biosensors and Bioelectronics* **2008**, 23, 1908-1914.
- (60) Spégel, P.; Schweitz, L.; Nilsson, S. *Electrophoresis* **2001**, 22, 3833-3841.