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COATINGS FOR CAPILLARY ELECTROPHORESIS

DOCTORAL THESIS

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I hereby declare that I have written the doctoral thesis myself. All used information and literary resources are indicated in the list of references.

In Olomouc ....…….

signature

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SUMMARY

Surface modifications of fused silica capillaries for use in capillary electrophoresis have been studied. Such coatings are essential in situations when it is necessary to prevent analyte adsorption that may lead to peak tailing and deterioration of the separation. They are also found useful for changing the direction or magnitude of the electroosmotic flow, or when interactions with the surface are desired for the purpose of chromatographic retention that influences the separation.

A number of various approaches have been introduced including dynamic or covalent modifications of the capillary surface determining its suitability for certain application. In this work, three demonstrations of capillary coatings for separation of small molecules by means of capillary electrophoresis are described.

For analysis of diastereoisomers of decarboxylated impurities related to *S*-adenosylmethionine a dynamic coating based on the background electrolyte containing barium borate was utilized. Such modification allowed to reduce the magnitude of the electroosmotic flow at alkaline pH and separation of the diastereoisomers with resolution of 1.35 was achieved in 10 minutes. The developed method allowed determination of the impurities in highly concentrated sample of *S*-adenosylmethionine without interference of the main component.

Separation of tartaric acid enantiomers with use of contactless conductivity detection was achieved in polyacrylamide coated capillary as it was necessary to suppress the electroosmotic flow directed against migration of tartaric acid. Complex of copper(II) and *trans*-4-hydroxy-L-proline was used as a chiral selector, resolution of 1.9 and limit of detection about 20 μM was achieved, however, baseline separation could be maintained only till enantiomeric ratio of 1D : 7L. Use of various counter-ions in the background electrolyte was studied and reversal of migration order of the enantiomers was observed. The method was applied on analysis of tartaric acid in wine, grape juice, and pharmaceutical substances containing tartaric acid as a counter-ion to the active substance. Only L-tartaric acid was detected.

The last part of the thesis is devoted to the fabrication of porous layer open tubular columns. An approach of delivering the capillary through a device equipped with LEDs for photoinitiated polymerization of porous polymers was pursued, however, even after modification of the polymerization mixture with a radical scavenger and a light-attenuating agent difficulties related with inhomogeneity in thickness distribution were still encountered. Optical profilometry was used together with scanning electron microscopy for assessment of the layer thickness. Improved results regarding the homogeneity of the coating were achieved with an approach based on replacement of the polymerization mixture by an immiscible liquid from the centre of the capillary followed by photoinitiated polymerization. Such procedure leads to formation of about 0.25 μm thick porous layer completely shielding the silica surface. Such modified capillaries were evaluated by anion-exchange capillary electrochromatography of selected inorganic anions enabling changing the migration order of iodide and nitrate by adjustment of the elution strength of the background electrolyte. Improvement in capacity factors over previously reported open tubular column was achieved, ranging from 8.9 to 20.1 %.

SOUHRN

V dizertační práci byly studovány modifikace povrchu křemenných kapilár pro použití v kapilární elektroforéze. Pokrytí stěny kapiláry je potřebné pro zabránění adsorpce analytů na povrch kapiláry, které může vést k chvostování píků a tím zhoršení separace. Dále jsou modifikace povrchu využívány pro změnu směru a rychlosti elektroosmotického toku, nebo pokud je žádána chromatografická retence pro ovlivnění separace.

V minulosti bylo vyvinuto množství způsobů pokrytí stěny křemenných kapilár, často jsou založeny buď na dynamické, nebo kovalentní modifikaci povrchu; vhodnost využití těchto způsobů závisí především na povaze použití. V této práci jsou představeny tři případy použití pokrytí povrchu s využitím pro separaci malých molekul kapilární elektroforézou.

Dynamické pokrytí využívající barnaté kationty a boritou kyselinu jako složky základního elektrolytu bylo využito pro separaci diastereoizomerů dekarboxylovaných nečistot *S*-adenosylmethioninu. Tato modifikace povrchu dovolila snížení rychlosti elektroosmotického toku v zásaditém základním elektrolytu. Separace diastereoizomerů s rozlišením 1,35 byla dosažena během 10 minut. Vyvinutá metoda byla použita pro analýzu této nečistoty ve velmi koncentrovaném vzorku *S*-adenosylmethioninu, jehož nadbytek nerušil při stanovení.

Kovalentní pokrytí polyakrylamidem pro eliminaci elektroosmotického toku bylo využito pro separaci enantiomerů vinné kyseliny s využitím bezkontaktní vodivostní detekce. Komplex měďnatých kationtů a *trans*-4-hydroxy-L-prolinu byl použit jako chirální selektor. Bylo dosaženo separace enantiomerů s rozlišením 1,9 s detekčním limitem přibližně 20 μM; nicméně separace na základní linii bylo možné dosáhnout pouze u vzorků, kde poměr enantiomerů byl nejvýše 1D : 7L. Použití různých protiiontů základního elektrolytu umožnilo změnit migrační pořadí enantiomerů. Metoda byla použita pro stanovení vinné kyseliny ve víně, hroznové šťávě a farmaceutik, která obsahují vinnou kyselinu jako protiiont k aktivní substanci. Ve vzorcích byl detekován pouze L-enantiomer.

Poslední část práce byla věnována přípravě *"porous layer open tubular"* kolon. Byly zkoumány možnosti protahování kapiláry skrz zařízení vybavené polovodičovými diodami (LED), které sloužilo pro světelně spouštěnou polymeraci pro modifikaci povrchu porózním

polymerem. Nicméně i při využití inhibitoru radikálové polymerace nebo látky zeslabující průnik světla do roztoku nebylo dosaženo uspokojivých výsledků co se týče homogenity distribuce tloušťky polymeru na stěně kapiláry. Kromě skenovacího elektronového mikroskopu byla využita také optická profilometrie pro zhodnocení tloušťky polymeru. Lepších výsledků bylo dosaženo promytím kapiláry kapalinou nemísitelnou s polymerační směsí zanechávající pouze tenkou vrstvu polymerační směsi na stěně kapiláry. Po její polymeraci bylo připraveno pokrytí o tloušťce přibližně 0,25 μm rovnoměrně pokrývající stěnu kapiláry. Modifikované kolony byly využity pro separaci modelové směsi anorganických iontů pomocí kapilární elektrochromatografie s anexovou stacionární fází. Změna eluční síly základního elektrolytu umožnila změnu migračního pořadí jodidů a dusičnanů. Bylo dosaženo zlepšení kapacitních faktorů oproti dříve připraveným *"open tubular"* kolonám o 8,9 až 20,1 %.

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1. INTRODUCTION

Coatings for capillary electrophoresis play an important role in its applications for analysis of various compounds when the surface properties and repeatability of its conditions of widely used bare fused silica capillaries are not found acceptable. Modifications of the capillary surface could be performed in a number of approaches utilizing large spectra of compounds dealing differently with specific problems as analyte adsorption or changing electrokinetic properties of the silica surface; or on the other hand mediating desired interactions with the surface as a stationary phase having an impact on selectivity of the separation.

Various additives as small molecules, polymers or particles have been introduced to alter separation parameters allowing e.g. much faster analysis by control over generation of the electroosmotic flow and its direction as well. A number of approaches have been developed for fabrication of porous polymer layers that enhance the surface area with different surface chemistries area allowing increased retention of molecules or more effective immobilization of various objects.

The necessity of characterization of the developed capillary coatings have brought attention to methods of assessment of coating properties and durability showing different applicability of the developed approaches for certain applications.

Several of the surface modification agents have been commercialized and are offered as a solution in specific situations, namely protein and peptide adsorption. Manufacturers of bare fused silica capillaries also offer already modified alternatives for selected applications.

2. THEORETICAL PART

2.1 Capillary electrophoresis

Electrophoresis is a separation method based on the migration of charged species in a supporting medium under the influence of an electric field. The ability of electrophoresis to separate charged species ranges from small inorganic or organic ions to charged biopolymers (like DNA or proteins), or even chromosomes, microorganisms, or whole cells [1]. The supporting medium is an electrolyte; in case of capillary electrophoresis (CE) it is placed into fused silica capillary that found use in other separation techniques as gas (GC) or liquid chromatography (LC).

After application of voltage to vessels to which ends of the capillary are immersed, introduced sample species migrate towards oppositely charged electrode. As various species possess different velocities, they are separated from each other and detected by a detector placed on the other end than the sample was introduced.

The velocity of an ion is given by an equation:

$$v = \mu_e E \tag{1}$$

where v is ion velocity, μ_e is an electrophoretic mobility and E is applied electric field. The mobility for a given ion, medium and temperature is a constant. The electrophoretic mobility depends on ion charge (q), medium viscosity (η), and ion hydrodynamic radius (r) by an equation:

$$\mu_e = \frac{q}{6\pi\eta r} \tag{2}$$

2.1.1 Electroosmotic flow

When electric field is applied, a flow of electrolyte solution is generated in the capillary. The flow is created by migration of ions compensating the charge of the capillary surface. In case of silica capillary acidic silanol groups are dissociated and cations from the electrolyte solution are attracted towards the surface to maintain electroneutrality. After application of voltage, these cations migrate towards anode together

with solvents molecules solvating the ion. That causes a net flow in the capillary and the phenomenon is called as the electroosmotic flow (EOF).

Electroosmotic mobility could be expressed by following equation:

$$\mu_{eo} = -\frac{\varepsilon\zeta}{\eta} \tag{3}$$

where ε is relative permittivity and ζ is zeta potential corresponding to charge of the surface. The magnitude of the EOF is governed on electrolyte pH and ionization of silanol groups.

The overall movement is therefore a result of both electrophoretic mobility and the EOF, it is expressed by apparent mobility which is calculated from the migration time of the solute:

$$\mu_{app} = \mu_e + \mu_{eof} = \frac{lL}{tV} \tag{4}$$

where l represents effective length of the capillary to the detector, L total capillary length, V applied voltage, and t migration time of the solute.

For separation of two sample components, resolution (R_S) represents the difference in migration distance and is calculated by:

$$R_S = \frac{2(t_2 - t_1)}{w_1 + w_2} \tag{5}$$

where t_1 and t_2 are migration time of maxima of the peaks, w_1 and w_2 are corresponding peak widths measured at baseline.

2.1.2 Modes of capillary electrophoresis

Capillary electrophoresis (CE) represents a family of techniques, all based on principle of electromigration, however, they differ in arrangement of the separation system [2].

Capillary zone electrophoresis (CZE) is characterized by a homogeneous electrolyte often called as the background electrolyte (BGE). A short plug of the sample is inserted and separation is obtained according to difference in mobilities.

Electrokinetic chromatography (EKC) implements a pseudostationary phase that is moving in the separation system with a different velocity than of analytes. They are resolved based on both migration and interactions mediated by the pseudostationary phase.

In capillary electrochromatography (CEC) the overall migration behavior is result of an interplay of chromatographic retention and electrophoretic migration, the stationary phase is also responsible for generating the EOF.

Isotachophoresis (ITP) utilizes electrolyte system of two electrolytes: leading and terminating. Leading electrolyte is composed of an ion with the highest mobility in the system whereas terminating electrolyte contains an ion with the lowest mobility of the separated mixture. Sample is introduced between the two electrolytes and its constituents form their individual zones behind the boundary of leading electrolyte. All zones are stacked to each other and move with the velocity of the leading ion. The concentration of the solutes changes after application of separation voltage and is given by Kohlraush regulation function [2].

Two other modes, capillary gel electrophoresis (CGE) and isoelectric focusing (IEF) are mostly used for separation of larger molecules as proteins or nucleic acids. CGE utilizes a sieving gel as a medium that enables separation according to size and shape, whereas in IEF the analytes are separated in a pH gradient according to their isoelectric points.

2.1.3 Detection techniques

Selection of a proper detection technique is largely dependent on properties of analyzed compound. Most often a spectrophotometric detection in near-UV region is utilized as it is provided in all commercially available instruments. However, not all compounds absorbs light at practically usable wavelengths, therefore other techniques have to be applied. Indirect UV detection based on displacement of an absorbing probe present in the BGE by a zone of analyte is one of the alternatives. As it uses the same detector it does not require any additional equipment, however optimal results are achieved when the mobility of the absorbing probe matches the mobility of the analyte. This is difficult to fulfill

for analysis of a mixture of different compounds, also introduction of another component in the BGE may influence equilibria and separation parameters.

Conductivity detection measures conductivity (or in other words displacement of the BGE co-ion with analyte with different mobility) between two electrodes, they could be in direct contact with the BGE, or alternatively they can be placed outside of the separation capillary in a contactless arrangement. Contactless conductivity detection (CCD) introduced by Zemann [4] overcomes limitations with contamination of electrodes, offers easy instrumentation and provides universal detection.

From other routinely employed detection techniques, laser-induced fluorescence detection (LIF) and mass spectrometry (MS) found use with electromigration techniques, especially when more selective and sensitive detection is required or in the case of MS identification is desired.

2.2 Capillary coatings

Coatings for fused silica capillaries have been introduced in order to shield capillary surface from undesired interactions, especially in analysis of basic compounds or proteins and peptides separation where peak broadening occurs due to wall adsorption. The other reason is to control the electroosmotic flow, which is essential for rapid, efficient and reproducible CE separation.

On the other hand when interactions with the surface are desired, choice of the stationary phase material plays essential role of its separation characteristics. The amount the stationary phase is comprising of the total column volume strongly determines its capacity as well as flow-through properties. Columns are often divided into three categories:

- open-tubular
- packed
- monolithic

2.2.1 Open tubular columns

In CE wall modifications leaving the profile of the capillary open are the most frequently used. Open tubular format allows easy washing and injecting into the capillary which is beneficial as most of CE instrument lack high pressure pump. A large number of chemical substances have been introduced, both dynamically or covalently attached onto the capillary wall.

2.2.1.1 Dynamic coatings

A large variety of compounds have been utilized as agents for surface modification simply by rinsing the capillary with their solution or by their presence in the BGE during analysis, depending upon strength of the interaction of the agent with the surface and the result expected [5]. Typically small molecules like diamines [6], cationic surfactants [7] and ionic liquids [8] could be added to the BGE in order to modify the surface properties.

When introducing coatings agents, another component of the BGE could raise absorbance, induce system peak [9] or its low volatility may interfere with electrospray ionization (ESI) for mass spectrometry (MS).

On the other hand, neutral or basic polymers could be adsorbed on the silica surface via several types of interactions as hydrogen bonding, Van der Waals interactions or coulombic forces [10]. The extent of interaction then determines stability and efficiency in preventing analyte-wall interactions. Derivatives of cellulose [11] or polyacrylamide [12] are mostly frequently used, followed by polybrene [13] or poly(diallyldimethylammonium chloride) [14] which also found use in formation of successive multiple ionic polymer layers [15] that are composed of sandwich-like structure of positively and negatively charged polymers.

2.2.1.2 Covalent coatings

Formation of permanent covalent bond between surface and the coating agent of capillary is another approach for surface modification of the capillary surface. Although more time and effort is required for preparation, improvement in lifetime and stability is usually obtained and such coatings allow changing the surface properties without presence of any additives in the BGE which is essential for CE-MS applications using ESI ionization.

Poor reactivity of silanol groups brings a necessity to introduce other functional groups that could be used further. Bifunctional silanes are mostly used to modify silica surface where the alkyloxy part is responsible for bonding with the silanol groups and the other functional group as e.g. amino [16], thiol [17], octadecyl [18] or perfluorinated alkyl chain [19] determines modified surface properties, or it can be used as an anchoring support for polymers. A large variety of monomers based on methacrylate [20], ethyleneglycol [21], or acrylamide [22] could be polymerized in-situ providing materials with diverse properties that can be further utilized, or already formed polymers as cellulose derivatives could be covalently attached to the surface [23].

2.2.1.3 Porous layer open tubular columns

Thin coating layers (in range of nanometers) often result in low capacity and thus hamper chromatographic applicability in case when large surface area is needed. Porous layer open tubular (PLOT) columns have been introduced to overcome the above mentioned limited capacity. The inner surface is coated with a stationary phase that has a porous microstructure, which greatly increases the effective surface area, thus significantly increasing the capacity to support interactions with solutes in the mobile phase. PLOT columns were first introduced to gas chromatography (GC) by Golay [24], and this format has become very popular for its significant improvements in efficiency and analysis speed over packed beds. Such columns are to date still produced by several manufacturers with numerous established applications [25].

Porous layer structures can provide high capacity due to their higher surface area when compared to non-porous polymeric coatings. Although the solid to liquid ratio for PLOT columns is lower compared to packed or monolithic columns of the same inner diameter, their capacities are still much greater compared to open capillaries and sufficient for many applications. By using narrower capillary diameters, acceptable efficiencies can be achieved [26]. As a principal advantage they show significantly higher permeability offering the possibility of rapid column flushing and regeneration, less demanding instrumentation requirements for pumping of solutions, and similarly to monolith and in difference to packed columns operation without the need to use frits.

2.2.1.3.1 Fabrication of PLOT columns

Significant progress in fabrication of PLOT columns have been made in recent years using different technologies and materials. A critical aspect lies in the ability to create the stationary phase at the capillary wall and prevent blockages in the centre. The means of controlling the process of its formation plays a crucial role and so far no general procedure has been reported that could be utilized for large range of materials regardless their chemical nature.

When a relatively thicker coating is desired, clogging is more likely to occur, and a careful selection and optimization of operating conditions has to be assured. However, stationary phases with a thickness in order of units or tens of μm are mostly desired that would show significant increase in the surface area compared to bare open-tubular capillary, while still keeping the advantage of good flow-through properties.

Surface properties of the capillary surface seem to be the governing factors of the formation of the stationary phase, as strong bonding to the wall is required; otherwise bleeding (loss) of the coating could occur. Recent works [27] showed challenges in preparing PLOT stationary phases in narrow diameter capillaries exhibiting different behavior as the smaller the diameter is, the greater role in the fabrication processes is played by the properties of the wall, affecting the properties of the stationary phase in terms of thickness as well as porosity.

Beside formation of polymeric or particle stationary phases, an alternative way how to increase the surface area is by etching with hydrofluoric acid, studied by Pesek [28]. 1000 times increase in the surface area of capillary wall was reported. More recently silica etching by supercritical water (Figure 1) was introduced by Karásek [29]. Such procedure allows to precisely tune the surface microstructure by varying the etching conditions together with an interesting possibility to control of capillary internal diameter along its length.

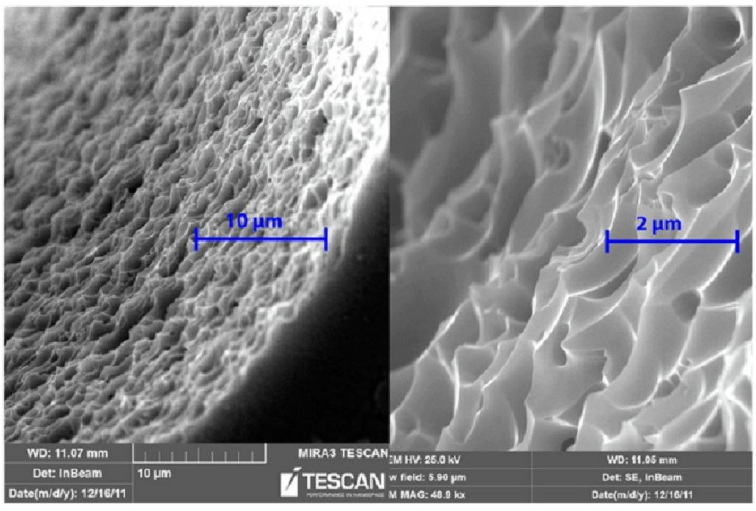


Figure 1. SEM images of silica capillary treated with supercritical water [29].

2.2.1.3.2 Sol-gel methods

Silica is the traditional separation medium for chromatographic methods finding place in most of packing materials and silica monoliths as well [30]. Such material possesses advantages of high mechanical stability, user defined pore size and tunable permeability related to porous structures. However, stability could be an issue when operating at extreme pH.

First porous open tubular column was reported about two decades ago, Tock *et al.* [31] exploited sol-gel process for preparation of a thin layer of porous silica. The procedure consists of gelation (by acidic or alkaline catalysis) of solution based on tetraethoxysilane (TEOS) or other alkyloxysilane that form an aqueous sol and the process results in a continuous network. Polycondensation and linking of additional silanol groups takes place, conditions as pH, temperature, and reagent concentrations control properties of resulting material. Formation of PLOT stationary phase consists in optimized conditions of pregeling step, mostly its time and temperature. However, obtained modification had poor capacity and retention of polyaromatic hydrocarbons [32].

Later Crego [33] improved the procedure to obtain thicker coating inside narrow-diameter capillaries by introducing ethanol into the reaction mixture to enhance TEOS solubility. C_{18}-modified 5 μm ID columns showed better performance, however, authors noted that lack of gradient elution and a availability of sensitive detection during their study.

A modified procedure was introduced by Guo and Colon [34] who applied pressure after the gelation process to remove the reagents from the capillary leaving only a thin film on the wall. Stability of the stationary phase was also tested showing resistance to both acidic and alkaline mobile phases; the authors reasoned that by using C_8-TEOS that has more hydrolytically stable Si-C bond than in case of usual silanization procedures based on Si-O bonds.

More recently, Forster *et al*. introduced a new generation of silica PLOT columns [35]. The reaction mixture is based on polyethylene oxide and urea in acetic acid solution with addition of tetrametoxysilane. Such mixture is stored in the capillary to allow in-situ

generation of ammonia responsible for formation of mesopores in the material. The authors noted significant influence of capillary diameter as in 50 µm ID aggregated particles were observed on one side of the wall caused by gravitation, but when narrower capillaries were treated a homogeneous layer was obtained (Figure 2). The influence of internal diameter between 10 and 20 µm was further studied [36] and the columns were applied for normal phase separation as well as using the reversed phase after modification with *N,N*-dimethylamino-dimethyl-octylsilane.

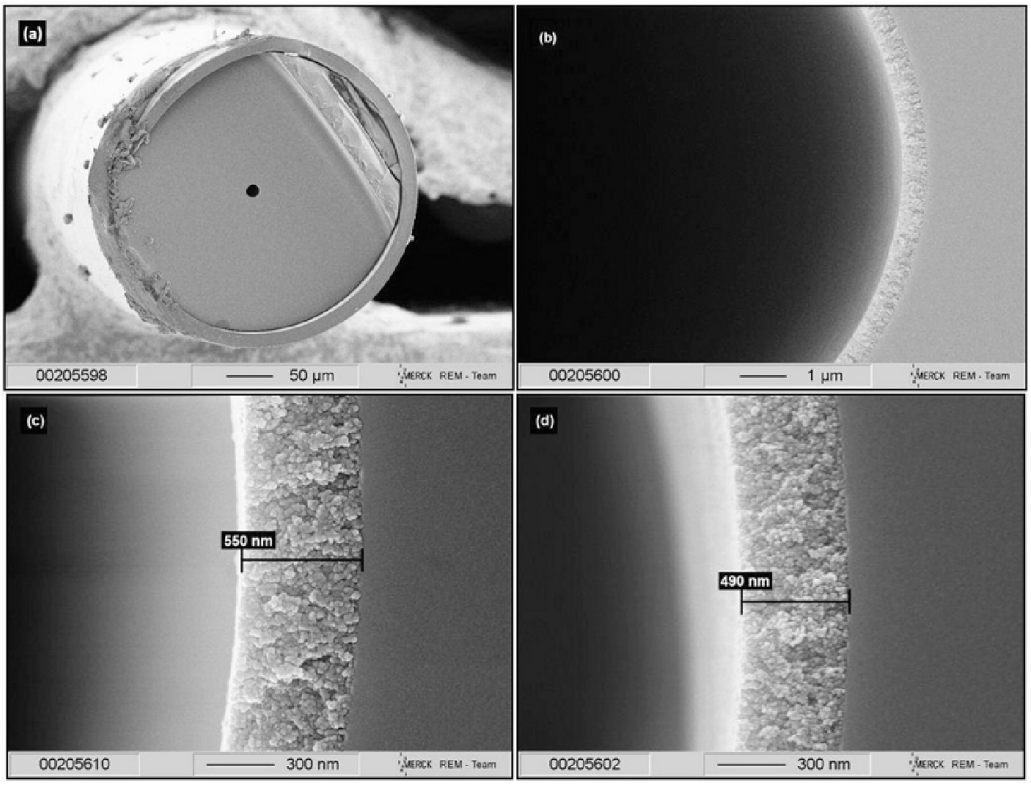


Figure 2. SEM images of silica PLOT column, bottom images show slight difference in layer thickness at opposite ends of the column [36].

Materials other than silica have been employed for fabrication of stationary phases as well, mostly zirconium and titanium oxide. Interesting PLOT column for enrichment of 5′-adenosine monophosphate was reported by thermal deposition of titanium oxide forming monolith-like structure on the capillary wall [37]. Metal-organic frameworks (MOFs) are attractive as novel separation medium due to their distinguished properties including large surface area, accessible tunnels and diverse structures. Aluminium oxide-based PLOT

stationary phase was synthesized by incorporation of particles into polymethylmethacrylate network and applied for separation of aromatic acids [38]. Another perspective lies in hybrid materials [39] produced by sol-gel processes combining advantages of easy in-situ preparation together with mechanical and chemical stability; however, no report of fabrication of PLOT column has been reported yet.

2.2.1.3.3 Porous polymers prepared by thermal initiation

Development of monolithic stationary phases in past two decades has led to exploration various materials and their utilization as a support in many chemistry challenges. Such porous polymers are mostly prepared exploiting thermally initiated radical polymerization, 2,2'-azobisisobutyronitrile (AIBN) is the traditionally used initiator [40-44]. Its thermal decomposition releases free radicals and by propagation reaction in presence of functional and linking monomers a polymer is formed. Solvents present in the polymerization mixture influence solubility of monomers and originating polymer and hence affect the resulting structure that exhibits porous properties with large surface area. When formation of PLOT monolithic column is desired, the polymerization process takes place regardless the position and thus it has to be controlled.

Dilution method

Typically, concentration of monomers for preparation of monolithic stationary phases is kept at 40% (v/v). When the content of porogenic solvents is increased over a critical ratio, the resulting polymer is not filling whole volume of the capillary. Although there are polymer nuclei present everywhere in the capillary, often those small nuclei in the inner lumen of the capillary are flushed away.

The formation of polymer is strongly dependent on chemical characteristics of used monomers as well as concentration of initiator and temperature. Due to many factors influencing polymerization kinetics, it is difficult to directly control mesoporous structure of resulting polymer. Irregularities regarding coating homogeneity could be observed due aggregating formed particles at one side of the wall caused by gravitation.

Despite the limitations, the dilution method has become popular in preparation of molecularly imprinted polymers (MIP). Methacrylic acid, trifluoromethacrylic acid, vinyl pyridines and acrylamides are frequently used functional monomers. All of these monomers interact with template functionalities via hydrogen donor-acceptor and/or coulombic interactions [40]. Such columns found applications in both LC and CEC separations of enantiomers, when the coating is imprinted with one of the enantiomers as in example of *S*-ketoprofen [41]. The authors also reported to improve the separation resolution by using 1 m long columns [42]. Furthermore, protein-bound PLOT columns prepared from a diluted polymer solution were reported for amino acid separations [43]. Even a single monomer, 2-methacrylamidopropyl methacrylate, was used for preparation of MIP for enantioseparation of amplodipine, naproxen, and ketoprofen [44].

Suitable porogens were found in aprotic solvents that do not interfere with the non-covalent binding between complementary functionalities. A ternary porogen system was developed [45], consisting of toluene, isooctane and dimethylsulfoxide, specifically designed to solve polar template of zopiclone and allowed the fine control of porogen properties of the resultant MIP coatings over a wide range of thickness (1–10 μm). An example of such column is shown on Figure 3. Huang [46] tested different ratio of monomers (hydroxyethyl methacrylate and 2-vinyl-4,4-dimethylazlactone) to 1-decanol and found optimal ratio of 1 : 6 providing about 1 μm thick porous layer that was later modified by bovine serum albumin for separation of amino acids.

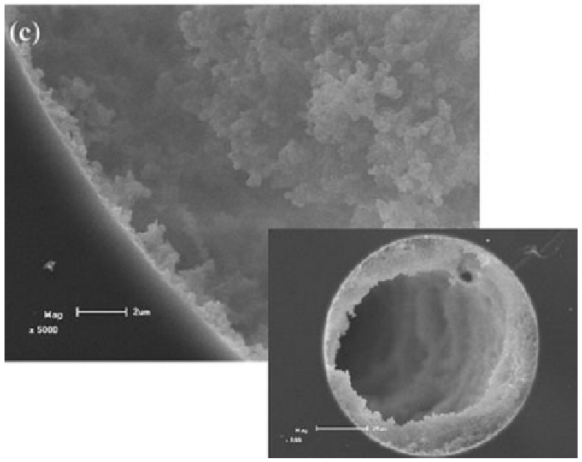


Figure 3. SEM image of MIP-coated capillary prepared with 30 % dimethylsulfoxide [45].

Short polymerization time

Typical reaction time for thermally initiated preparation of monolithic stationary phases is ranging from 20 to 24 hours to ensure that all components underwent polymerization and sufficiently reproducible product is obtained. Decreasing time the reaction is allowed to progress then leads to formation of shorter polymer chains. Although such process is more difficult to control, there are reports on the increase of efficiency when polymerization is not completely finished [47].

PLOT columns could be obtained by empirical development that allows applying such a method for any composition of stationary phase. Such procedure is rather useful for fabricating thin coatings as longer reaction time increases a risk of undesirable crosslinking or attachment of clusters formed in the centre of the capillary. Tan [48] studied an effect of polymerization time on the performance of the prepared column on enantioseparation of dansylated phenylalanine by LC and CEC, best results were obtained with polymerization time of just 10 miunutes. Combination of dilution of monomers and reducing the polymerizaton time to 4 hours was demonstrated by Zaidi [42].

Confinement

The surface has a fundamental effect on the formation of the polymer which is in contact with it. It often causes differences in morphology from the bulk polymer up to a few microns into the bulk volume, an effect that becomes more important as the environment becomes more confined. In most of cases the capillary surface is silanized by γ-methacryloxypropyltrimethoxysilane (γ-MAPS) in order to anchor formed polymer structure to the wall. Such modification strongly changes the surface properties. Gibson [49] studied effects of surface modification of the fused silica capillary wall on the thickness and morphology of the sheath surrounding the porous polymer. They found a correlation between contact angle of the capillary and connection between the porous polymer based on butyl acrylate and 1,3-butanediol diacrylate, that with rising hydrophobicity less connections to the sheath are made.

Structural differences with decreasing capillary diameter were observed by He *et al.* [27]. A shift from forming typical monolith structure filling whole volume of the capillary to presence of the polymer layer only at the capillary wall was shown as displayed on Figure 4. Authors suggest that the deformation of the structure results from the wall effects like wettability of polymerization mixture constituents. The smaller the ratio of capillary to bulk monolith pore size, the greater is the difference of the product morphology from a bulk polymerization product. Deviation from the bulk porous structure occurs when the confining dimensions are within a factor of 5–10 of the bulk pore size of the monolithic material. As the confinement dimension decreases, the uniformity of polymer microglobule dispersion is decreased [50]. Also, polymerization kinetics and related diffusivity of formed nuclei is involved as by manipulating with capillary diameter the diffusion time from the centre of the capillary to the wall dramatically differs when 5 μm and 25 μm ID capillaries are used [27].

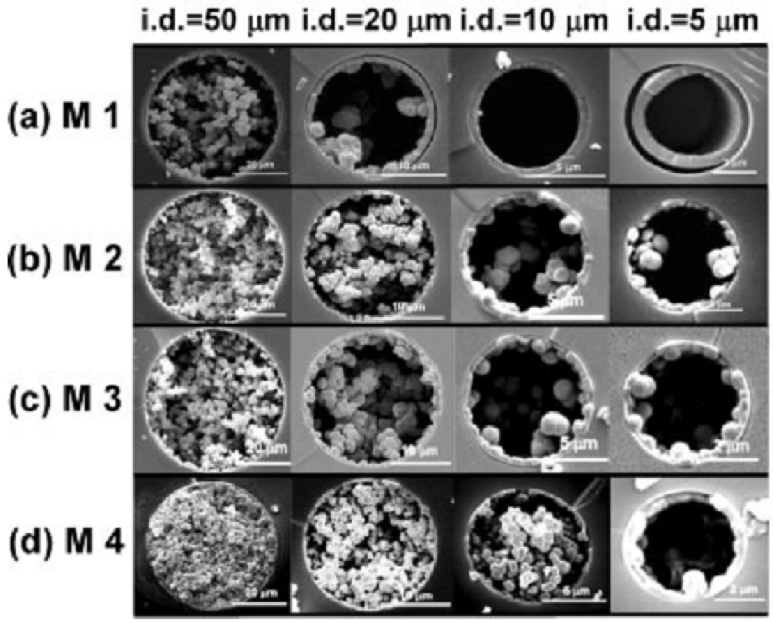


Figure 4. An effect of different capillary ID on resulting polymer morphology for various polymerization mixtures leading into different macropore size [27].

Similar results of producing PLOT columns in narrow diameter capillaries have been achieved and successfully applied by other groups: Huang prepared vinylbenzyl chloride and divinylbenzene PLOT column in 20 μm capillary which was further modified with *N,N*-dimethyldodecylamine for separation of proteins and peptides. Group of Karger has investigated use of 4.2 metres long 10 μm ID PLOT poly(styrene-*co*-divinylbenzene) (PS-DVB) columns [51] or more recently ethylenediamine modified poly(vinylbenzyl chloride-*co*-divinyl benzene) [52] for LC-MS proteomic analysis. Narrow diameter columns were also found advantageous for direct coupling to MS due to increased ionization efficiency of nanoelectrospray. Rogeberg *et al.* followed previous fabrication method and separated intact proteins [53]. Later, the same group used two PLOT columns first for enzymatic cleavage of proteins with trypsin modified poly(2-hydroxyethyl methacrylate-*co*-vinyl azlactone) 20 μm ID column followed by separation on previously used 10 μm ID PS-DVB column [54].

Such demonstrations of using confinement effects clearly show that such method is applicable for preparation of PLOT columns from various polymerization mixture differing in hydrophobicity of their constituents. Even though the porosity does not provide dramatic

increase in the surface area, the narrow capillary diameter reduces issues of slow mass transfer and diffusion in the liquid phase.

Laminar flow

In contrary to confinement effects in narrow capillaries, effect of different fluid velocities in laminar flow conditions was exploited by Collins [55] in 50 μm columns or larger. Thermally initiated polymerization takes place at the capillary wall due to heating the liquid in contact with surface and also the faster flow in the central part of the capillary drags formed polymer out. However, the flow rate is limiting factor as with speed higher than 1 mm/s a nonporous layer was obtained. Polymerization mixtures based on butyl methacrylate and ethylene dimethacrylate and PS-DVB were tested, both showing a possibility to control the thickness of the coating that was on-line monitored by contactless conductivity measurements.

Surface initiated atom transfer radical polymerization

In an ideal situation of PLOT column fabrication, the polymer is growing from the capillary wall and therefore any irregularities caused attachment of polymer from the capillary centre are avoided. In surface initiated atom transfer radical polymerization (SI-ATRP) the initiator is immobilized onto the surface and the thickness is controlled by time the process is allowed to proceed [56].

Polyacrylamide film was deposited by Huang [57] and influence of polymer crosslinking was demonstrated on separation of basic proteins. Schweitz [58] prepared MIP coating with thickness ranging from 0.15-2 μm.

2.2.1.3.4 Porous polymers prepared by photo-initiation

Polymerization of porous materials initiated by light-sensitive compounds is another attractive option in preparation of PLOT columns. Usually the process is much faster than in the case of thermal initiation, but the radiation dosage could be more precisely controlled.

However, most of photo-initiators as 2,2-dimethoxy-2-phenylacetophenone (DMPAP), benzophenone, and benzoyl peroxide absorb at UV-region, this approach is applicable only for transparent materials which bring necessity to use Teflon-coated silica capillaries which are more expensive and fragile than mostly used polyimide-coated. From the material point of view, preparation of PS-DVB or similar stationary phases is not possible due to strong UV absorbance. Nevertheless, a possibility of photografting in the second step of fabrication makes the developed procedures applicable for any surface chemistry desired.

Capillary rotation

First PLOT column prepared using photo-initiation was reported by Eeltink [59] who rotated the capillary at 100 rpm below a UV-light source in order to provide equal dosage on the surface. Prepared poly(butyl methacrylate-*co*-ethylene dimethacrylate) was photografted with acrylamido-2-methyl-1-propanesulfonic acid to generate fast EOF for CEC separation of alkylbenzenes, the SEM image of the column is shown on Figure 5. The authors used diluted polymerization mixture of 2% monomers to better control the thickness. Such approach also takes advantage of centrifugal force which pushes polymer particles growing from the capillary centre to the wall and thus prevents clogging when thicker coating is desired. However, the length of the column is limited by the length of illuminated area.

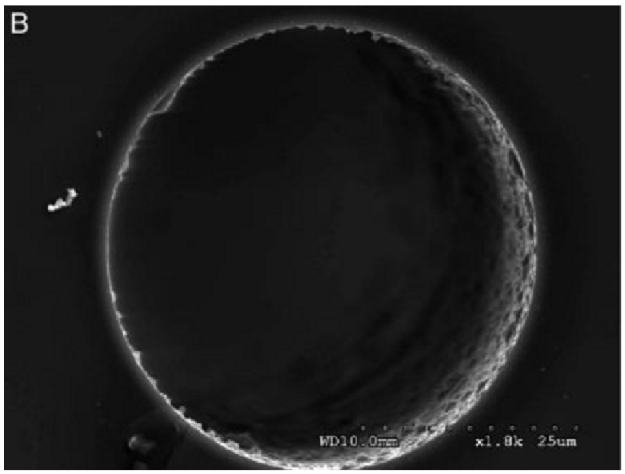


Figure 5. SEM image of capillary coated prepared with UV irradiation and revolving at 100 rpm from mixture of 2 % butyl methacrylate, 2% ethylene dimthacrylate, 96 % 1-octanol, and 0.1% DMPAP [59].

Evanescent wave

Capillary illuminated axially produces an evanescent field at the inner wall of a transparent polytetrafluoroethylene-coated (outside coating) fused silica capillary and photoinitiated polymerization occurs mostly at the capillary wall. Such approach was exploited by Abele [60] for fabrication of PLOT enzymatic microreactor for on-line protein digestion. It was found that the procedure was not applicable to polyimide coated due to its higher refractive index than of silica. The rapid decay of evanescent field intensity limits the column length that could be prepared (approximately 7 cm).

Scanning

Longer PLOT columns were prepared by Nesterenko *et al.* [61] by repeatedly passing light source over the capillary. When the capillary was placed on a highly-reflective surface, homogeneous coating of poly(glycidyl methacrylate-*co*-ethylene dimethacrylate) (GMA-EDMA) had been obtained. Surface epoxy groups were hydrolyzed to diols. Prepared column was applied for separation of test mixture of proteins with isocratic elution.

2.2.2 Packed columns

Packed columns for CEC are an extension from separation media widely used in LC. Most often slurry of silica or organic polymer particles is packed into the column using high pressure until the column is filled between beginning and end frits are made using sintering processes; end part of the capillary is left open for on-column optical detection [62]. The advantage of packed column lies in the fact that a number of existing LC stationary phases can be used with a range of various retention properties and well-described selectivity. That should make the method development in CEC easier, however, different separation selectivity of CEC from LC especially for charged analytes must be kept in mind [63].

2.2.3 Monolithic columns

Stationary phases based on a continuous bed of porous polymer were explored after development of column-packing technology. The monolithic column can be made from many different materials, most often from silica or organic polymers. When compared to packed columns, monolithic columns became popular for higher permeability, low mass transfer resistance, simple in-situ preparation, and no frit requirement [64]. A large number of monomers possessing various functional groups are available or the chemical properties of the material could be tailored using grafting processes. That is advantageous for introduction of charged groups for ion-exchange interactions and the EOF generation which are responsible for polymer swelling [65].

2.2.4 Characterization of columns

Many different approaches have been developed and introduced for characterization of capillary coatings and stationary phases in CE. Assessment of the EOF regarding its direction and magnitude in various BGEs, repeatability or stability during a given number of runs or time are mostly performed as only injection of suitable neutral EOF marker (dimethylsulfoxide, acetone, thiourea, etc.) is necessary.

Testing of analysis performance of separation of model compounds may provide more valuable data regarding applicability of the coating for certain applications and allows comparison between different approaches. Gomez [66] used a cationic probe, $Ru(bpy)_3^{2+}$, that strongly interacts with silanol groups. Peptides and proteins have been frequently used as testing analytes, peak asymmetry and width [67] or protein recovery based on quantitation with two detectors [68] or capillaries with different length [69] have been reported as parameters in capillary coating studies.

Evaluation of stationary phases in LC is usually based on retention characteristics of set of model compounds and related capacity of the column. Spectral methods could provide valuable information in a non-destructive way as in case of infrared spectroscopy [70,71] used for inspection of surface modification with polymers or nanoparticles through a window made in polyimide coating further used for optical detection.

On the other hand, physical properties of the material could provide valuable data, therefore measurement of specific surface via nitrogen adsorption or pore volume and pore size distribution by mercury intrusion are widely used [72]. However, these techniques could be applied only to a larger amount of material that has to be prepared in bulk, therefore obtained results could differ from products prepared in much more confined vessels as capillaries with ID of tens of μm [27]. In such case inspection by microscopy techniques allows visualization of the prepared product directly inside the capillary.

2.2.4.1 Scanning electron microscopy

Scanning electron microscopy (SEM) is frequently used for measurement of size and size dispersity of elements. Electrons generated by an electron gun are accelerated and focused on the sample, the primary electrons cause low energy secondary electrons to be emitted and some of them escape the sample surface. The secondary electrons are detected and by scanning the primary electron beam across the sample an image is recorded. A SEM setup is shown on Figure 6.

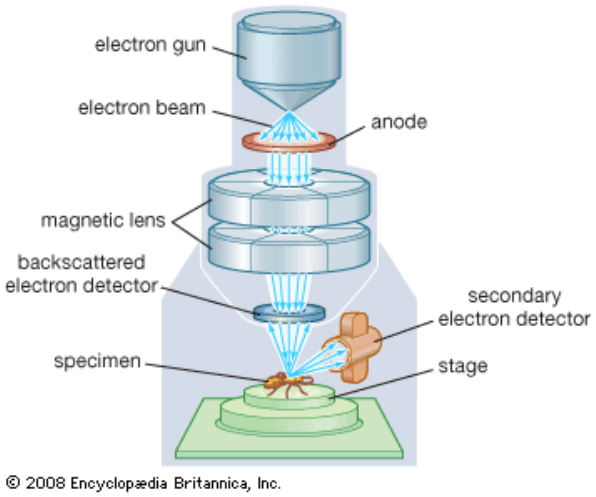


Figure 6. A scheme of SEM instrument [73].

As SEM operates in vacuum, sample has to be dried and if it is not conductive it has to be eventually sputtered by a thin layer of metal coating to prevent charging of the sample that may disturb the quality of the obtained image. SEM offers magnification in order

from hundreds to hundred thousands and even millions could be achieved with recent instruments revealing details smaller than 1 nm. Another advantage lies in a large depth of field that is useful for visualization of surface structure. However, SEM allows characterization in vacuum which does not reflect situation in liquid phase when swelling of polymers may occur at various extent. Other techniques as atomic force microscopy [74] or laser scanning confocal microscopy [27] can operate when the material is immersed in the mobile phase used during separations and provide deeper insight into material morphology that SEM is not capable of.

2.2.4.2 Optical profilometry

Optical profilometry or in other words coherence scanning interferometry serves as non-contact method for study of surface morphology to obtain three-dimensional measurement.

The technique is based on the principle of constructive and destructive interference depending upon phase shift between two phases. In an instrument (a typical design and operating principle is shown on Figure 7) light from a light source (could be both monochromatic and white) is split by a beam splitter in two parts that travel different paths - one is reflected from an internal reference mirror, the other one interacts with the sample. The two waves are then combined to create interference and interference fringes are monitored on the image sensor. By vertical scanning of the sample an interference dependence height on is obtained.

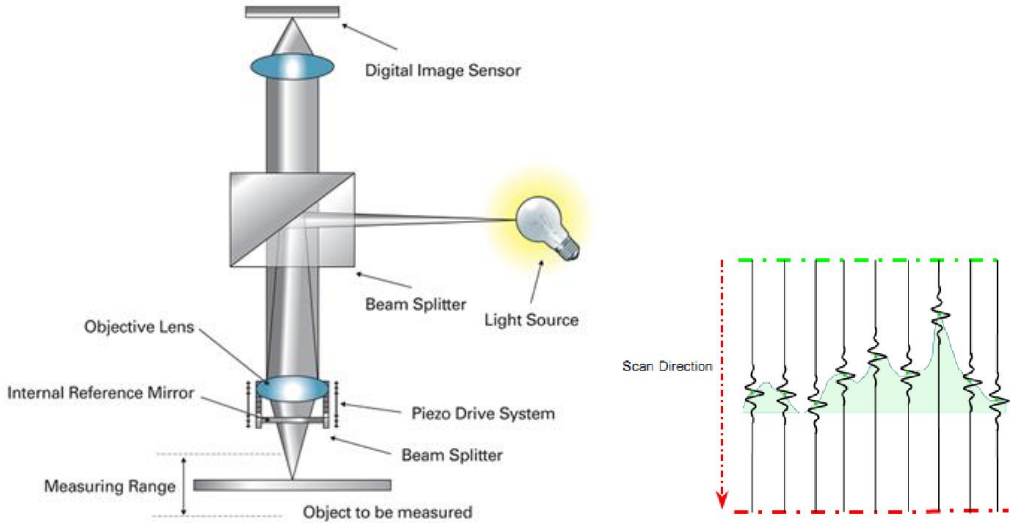


Figure 7. A scheme of optical profiler instrument (left) and a scheme of interferences observed at different sample profile elements with different height (right) [75].

Optical profilometry finds its use in evaluation of surfaces regarding their flatness, roughness or film thickness and also is widely applied for assessment of microfabricated devices in microfluidics, micro-electro-mechanical systems (MEMS). A representative image obtained by optical profilometry of a microfluidic device is shown on Figure 8.

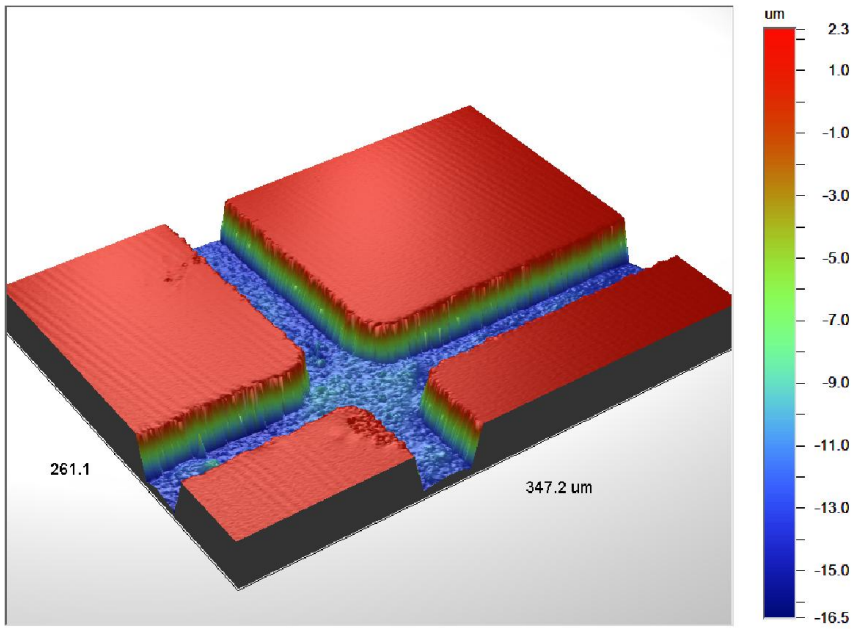


Figure 8. An illustrative image of a cross-section of polydimethylsiloxane microfluidic device obtained by an optical profiler.

3. AIMS OF THE THESIS

The aim of the thesis was to explore possibilities of employment of various capillary coatings in applications of capillary electrophoresis to both reduce and support interactions with the capillary surface. Such modifications should be used in order to improve analysis performance regarding analysis time and resolution of analytes.

The specific aims of the thesis could be divided into three parts:

i. Apply capillary coatings in order to prevent adsorption of basic compounds as derivatives of *S*-adenosylmethionine to prevent peak tailing.
ii. Apply covalent capillary coatings for control of the electroosmotic flow direction and magnitude in separation of tartaric acid enantiomers .
iii. Explore possibilities of formation of a stationary phase for capillary electrochromatography with increased surface area over classical open tubular format.

4. RESULTS AND DISCUSSION

4.1 Analysis of decarboxylated products of *S*-adenosylmethionine

Results shown in the section have been published in the following article:

- R. Sebastiano, R. Knob, A. Citterio, P.G. Righetti, *Analysis of trace degradation products (decarboxylated diastereoisomers) of S-adenosylmethionine by electrophoresis in capillaries with cationic coatings (N-methylpolyvinylpyridinium or divalent barium)*. Electrophoresis 31, 2010, 3592-3596.

4.1.1 *S*-Adenosylmethionin

S-Adenosyl-L-methionine (SAM) belongs to group of biologically active compounds primarily responsible for transfer of a methyl group during metabolic processes, its structure is shown on Figure 9. In fact, it is the only sulfonium cation present in mammals‘ body, SAM takes part in numerous methyltransferase reactions aimed on modification of various types of metabolites and macromolecules as proteins, nucleic acids, and polysaccharides.

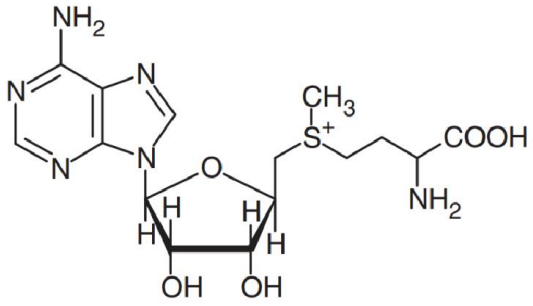


Figure 9. Structure of *S*-Adenosyl-L-methionine.

SAM exists in two diastereoisomeric forms, *S,S*- and *R,S*-adenosylmethionine, where the designations refer to the stereochemical configurations of the sulphur and the α-carbon. Only the *S,S*-form is biologically active; in mammals there is only a minor fraction of the *R,S*-SAM [76]. SAM acts as a methyl group donor, *S*-adenosyl-L-homocysteine (SAH) is produced in the transmethylation reaction and simultaneously acts as an inhibitor

of the process. SAM can be decarboxylated to *S*-adenosyl-(5)-3-methylthiopropylamine (dcSAM) and even this compound can exist in two diastereoisomeric forms. The structures are shown on Figure 10. These diverse forms of heterogeneity have called for methods for the rapid and accurate determination of SAM and of possible metabolites.

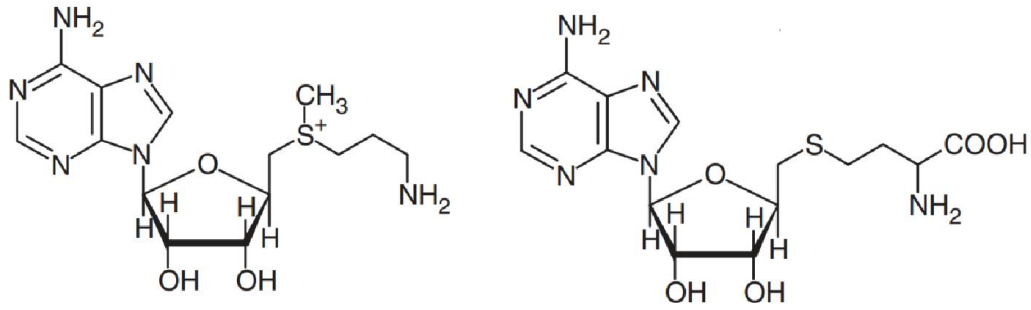


Figure 10. Structures of *S*-adenosyl-(5)-3-methylthiopropylamine, dcSAM (left) and *S*-adenosyl-L-homocysteine, SAH (right).

4.1.2 Analysis of *S*-adenosylmethionin degradation products

Many studies have been performed on the assessment of SAM stability and the hydrolytic processes that occur under the biological experimentation. Parks [77] and Baddiley [78] studied the hydrolysis of SAM and observed the formation of adenine under acidic conditions and methylthioadenosine and homoserine by heating in neutral solution.

Disorders in SAM metabolism have been reported in several diseases [79-81]. Commercial preparations of SAM derived from yeast cells often contain SAH which then acts as the inhibitor of transmethylation reaction. Method was analysis of SAM and its metabolites along with natural polyamines was developed by Wagner [82] using reversed phase ion pair HPLC. Similar study using cation-exchange chromatography was introduced by Zappia [83] and allowed measurements of enzyme activities involving compounds mentioned above.

Capillary electrophoresis has been utilized for separation of SAM and SAH in an uncoated capillary using 40 mM sodium phosphate (pH 4.0) buffer [84]. The same compounds were determined in mouse and rat tissues using 200 mM glycine hydrochloride BGE at pH 1.8 [85]. Rate of SAM hydrolysis incubated at 38 °C was assessed [86]

demonstrating the suitability of CE over HPLC for fast and more efficient monitoring of the process.

Separation of dcSAM diastereisomers was reported by Dejima *et al*. [87] who used two dimensional reversed phase ion pair HPLC with preparative and analytical columns with isocratic elution by 0.36 M sodium phosphate pH 4.0 containing 5 % (v/v) acetonitrile, 8 mM sodium octanesulfonate, and 0.1 mM EDTA. *S*-dcSAM was the first eluting diastereomer. However, the separation was achieved in about 55 minutes. In the following study, aim of the work was focused on separation of decarboxylated products of SAM by capillary electrophoresis in order to provide adequate method for rapid production control of SAM.

4.1.3 Experimental conditions

Formic acid, boric acid, ammonium hydroxide, sodium hydroxide, barium hydroxide, and acrylamide were obtained from Fluka. HPLC grade water, hydrobromic acid, hydrochloric acid, methanol and propanol were obtained from Sigma-Aldrich. Barium carbonate was prepared by precipitating barium hydroxide with carbon dioxide (dry ice), the precipitate was filtered, washed and dried. SAM sample in form of *p*-toluenesulfonate and 5'-methylthioadenosine (MTA) were obtained from Omniabios S.r.L., Bagnolo Mella, Brescia (Italy).

The analyses were performed by an HP^{3D} CE equipped with an UV-Vis diode-array detector. Fused silica capillaries of 50 μm ID were purchased from Composite Metal Service (United Kingdom). Total capillary length was 32.5 cm, effective length was 24 cm. All flushing was performed with assistance of external pressure (4.5 bar), temperature of the capillary cassette was set to 25 ºC. Injection of samples was performed by application of pressure of 30 mbar for 5 seconds followed by injection of the BGE at 5 mbar for 2 seconds. Detection was performed at 254 nm unless stated otherwise.

All new capillaries were rinsed with 1 M NaOH followed by water; *N*-methylpolyvinylpyridinium (PVPy-Me) modified capillaries were prepared by rinse with a solution of 1 mg/ml PVPy-Me in water for 3 minutes followed by the BGE for 3 minutes.

For analyses with the BGE based on barium borate the capillary preconditioning consisted of 0.5 min flush with water, 0.5 min with 0.1 M HCl, 0.5 min with water, and 1.5 min with the BGE. At the end of the day the capillary was washed with 0.1 M HCl for 5 minutes and left in water overnight in order to prevent deposition of barium carbonate. Similarly, the electrolyte vials were washed with 0.1 M HCl to dissolve and remove any traces of barium containing compounds.

4.1.4 Separation using uncoated capillary

In the first experiments in an uncoated capillary, ammonium formate based BGE was selected for separation of dcSAM from the main sample component, SAM. The BGE was chosen reflecting further aim to transfer the method for CE-MS experiments in order to confirm peak identity with data obtained from MS. As separation of only diastereisomers of dcSAM was desired, no chiral selector was added into the BGE as separation of enantiomers producing up to 4 signals could be expected. Due to absence of standards it would be impossible to assign identity of related signals to corresponding diastereisomeric forms of dcSAM.

Analysis of the SAM sample at concentration of 5 mg/ml in 100 mM ammonium formate pH 4.0 is shown on Figure 11. Beside peaks of dcSAM (which was confirmed later by standard addition) and SAM, multiple peaks were observed, due to their much smaller relative peak area compared to peak of dcSAM they were not studied further. Peak of dcSAM looked as a single symmetrical peak, therefore no hint of diastereisomers separation was observed. BGEs based on ammonium acetate with higher pH were tested to reduce effective mobility of dcSAM and possibly allow separation of the diastereisomeric forms which could only slightly differ in their electromigration behaviour.

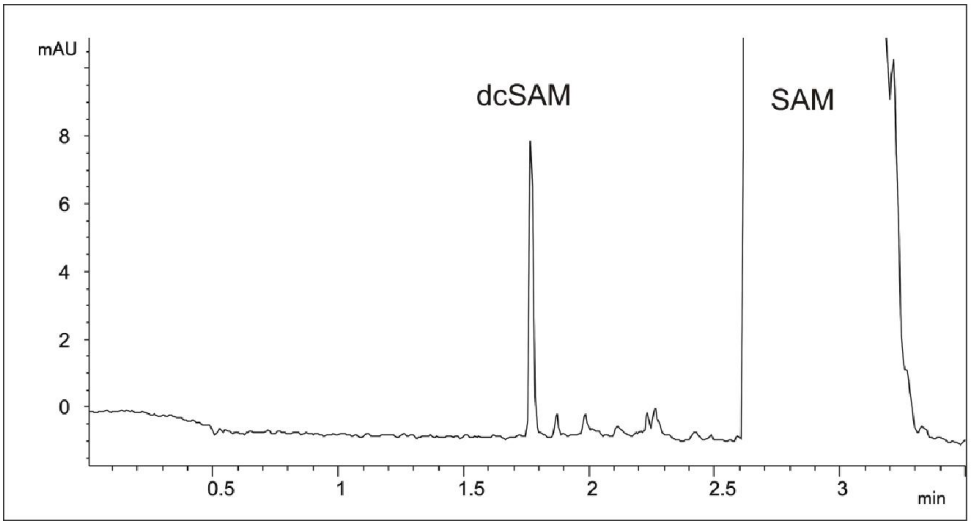


Figure 11. Analysis of SAM sample at concentration 5 mg/ml in 100 mM ammonium formate pH 4.0 using uncoated silica capillary.

In the BGEs with pH higher than 6.0 the resolution between dcSAM and SAM peaks increased, however, due to strong interaction of these basic compounds with silanol groups of the silica capillary the separation became more deteriorated due to significant peak tailing.

4.1.5 Modification by *N*-methylpolyvinylpyridinium

In order to decrease effective mobilities and provide longer separation time for separation of dcSAM diastereoisomers, modification of the capillary wall by cationic polymer, *N*-methylpolyvinylpyridinium (PVPy-Me), was introduced.

PVP-Me iodine was available as in-house synthesized [88]. Such polymer has been previously successfully applied for separation of both low and high molecular weight proteins with various p*I* values and improved run-to-run repeatability [89].

The surface modification led to deposition of positively charged polymer on the capillary wall with the result of reversing the EOF direction towards anode. Using 50 mM ammonium formate pH 4.0 as the BGE, the electroosmotic mobility was determined as -32.5×10^{-9} m^2/Vs, measured at -20 kV with acrylamide as the EOF marker. Analysis of SAM sample at concentration 5 mg/ml is shown on Figure 12. The prolongation the migration time allowed the diastereisomers to partly separate as apparent from the doublet

peak between 9 and 10 min. However, the large excess of SAM migrating in this case in front of dcSAM competed with the cationic polymer coating on the capillary surface (it was necessary to reapply polymer coating procedure to obtain repeatable results) and thus diminished separation of the diastereoisomers due to observed peak tailing.

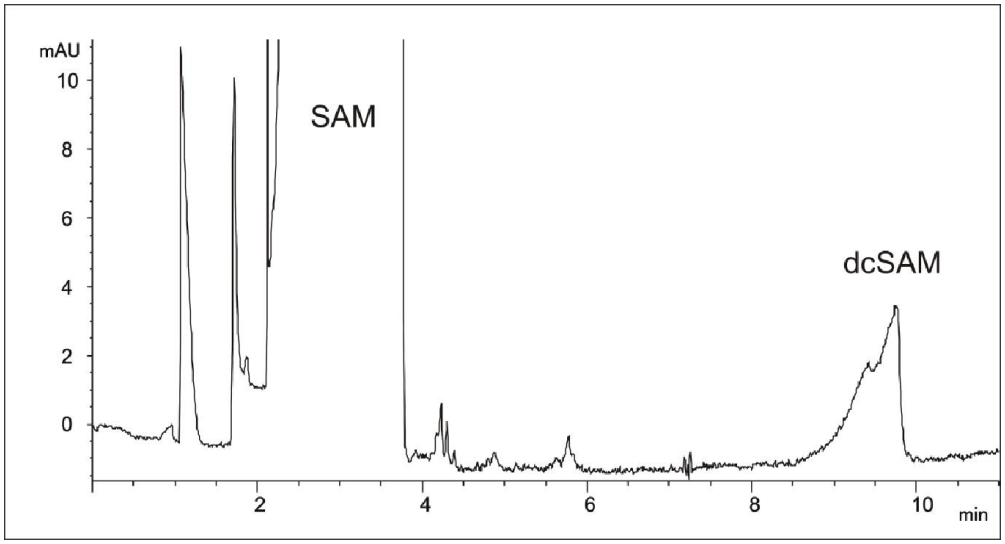


Figure 12. Analysis of SAM sample at concentration 5 mg/ml in 50 mM ammonium formate pH 4.0 using PVPy-Me modified capillary.

4.1.6 Synthesis of *S*-adenosyl-(5')-3-methylthiopropylamine

In order to confirm the peak identity, a standard of dcSAM was synthetized according to following procedure: 10.47 g of 5'-methylthioadenosine (MTA, obtained from Omniabios S.r.L., Bagnolo Mella, Brescia, Italy) was dispersed in 200 ml of 0.1 M HBr and 11.56 g of 1-bromo-3-aminopropane hydrobromide was added. Figure 13 shows the reaction. The mixture was left under stirring at 70 °C for three days, every 24 hours a sample was taken and analyzed by the developed CE method to monitor the progress of the reaction as shown on Figure 14.

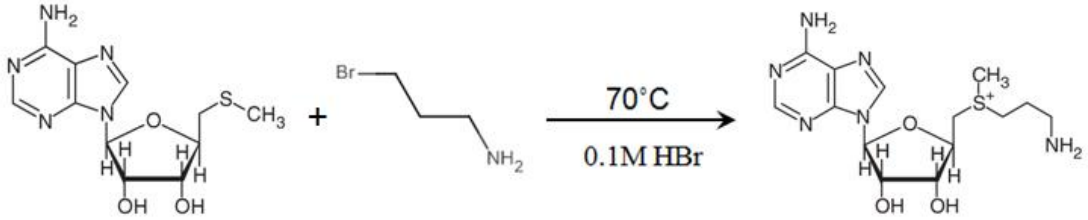


Figure 13. Reaction of dcSAM synthesis from 5'-methylthioadenosine (MTA) and 1-bromo-3-aminopropane.

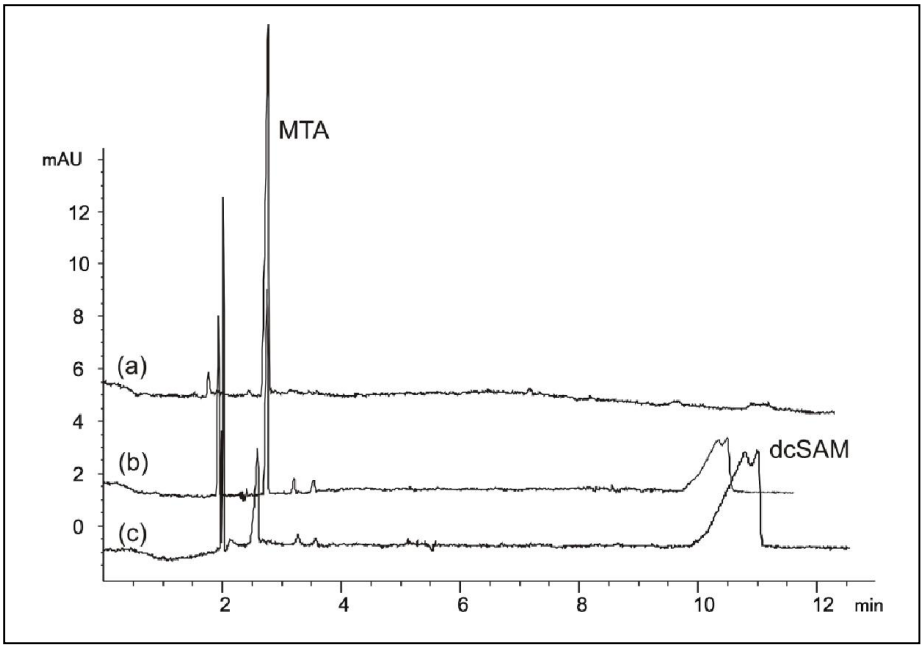


Figure 14. Monitoring of the reaction mixture (20 times diluted in the BGE) for synthesis of dcSAM using PVPy-Me modified capillary and 50 mM ammonium formate pH 4.0.
(a) Analysis at start of the reaction, only peak of MTA present.
(b) Analysis after 24 hours, peak of dcSAM appears.
(c) Analysis after 72 hours, peak of MTA was substantially decreased whereas dcSAM content increased. Side products of the reaction were not identified.

After no increase of the dcSAM peak area was observed, the obtained solution was evaporated a rotary evaporator. The residue was partially dehydrated by stripping to the rotary evaporator for several times with 10 ml of acetone, afterwards it was dissolved in 30 ml of methanol. The solution was added drop by drop to 200 ml of ethanol under vigorous stirring to obtain a precipitate. The mixture was filtered and the solid was washed several

times with 5 ml of ethanol. The solid was dried by a mechanical vacuum pump, the product was stored at 4 °C. The identity of product was confirmed by NMR and MS analysis (data not shown). Analysis of the purified synthetic dcSAM standard using PVPy-Me coated capillary is shown on Figure 15.

To assess the number of hydrobromic acid molecules present as couterions of dcSAM (in order to know molecular weight of the obtained product for later quantitation), content of bromide in the prepared synthetic dcSAM sample was determined by CE as 38.9 % (using an uncoated capillary, 50 mM ammonium formate as the BGE, voltage -20 kV). Such result suggests that the product was isolated in form of dcSAM . 3 HBr (corresponding theoretical bromide content would be 40.1 %).

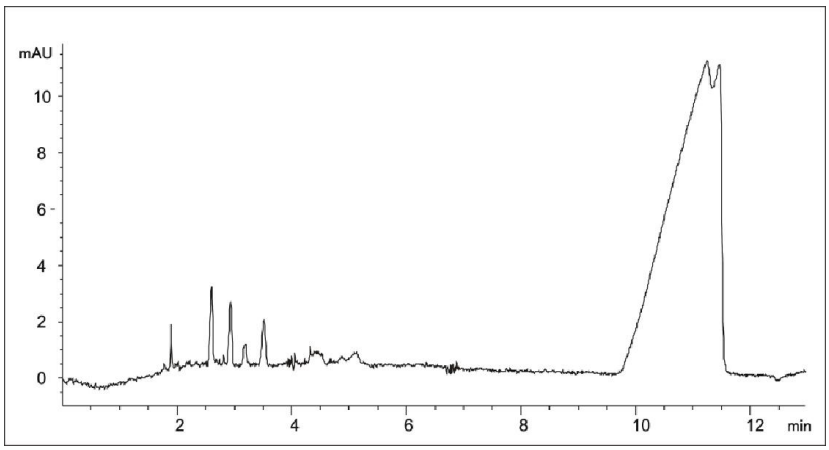


Figure 15. Analysis of dcSAM purified synthetic product (0.1 mg/ml), conditions same as in Figure 14. Based on relative peak areas, dcSAM could be declared as 96 % pure; 5 impurities were found, the most abundant one (peak at about 2.5 min) possesses 1.2 % of overall peak area.

When compared to Figure 12, the ratio of peak heights of the two partly separated signals differs from the SAM sample. That suggests non-equal formation of *S,S*- and *R,S*-dcSAM diastereisomers in contrary to non-stereoselective synthesis described above leading to ratio of the isomers of 1:1 - as confirmed by analysis of the product. However, insufficient separation of the isomers did not allow precise quantitation; therefore improvement by changing the experimental conditions was required.

Dynamic coating with another in-house prepared coating agent, 1-(4-iodobutyl)-4-aza-1-azoniabicyclo[2,2,2] octane iodide (M7C4I [90]), was tested, however, similarly as in case of covalent coating with polyacrylamide (described in following section 4.2.3.1) the surface

modification did not suppress the peak tailing observed in analysis of SAM sample ruining the separation of the diastereoisomers.

4.1.7 Barium borate modification

In another approach, dynamic modification with the BGE based on barium borate was examined. Barium salts were noted as effective additives to suppress the EOF even at higher pH values [91]. More recently, the BGE based on boric acid and barium hydroxide was utilized for separation of tryptic digest of proteins at pH from 8.5 to 11 with reversal of the EOF [92]. The authors came with a hypothesis formation of a sandwich-like structure at the capillary surface, where Ba^{2+} ions are adsorbed on negatively charged of silanol groups. Excess of the positive charge is compensated by borate anions partially precipitating into barium borate covering the original silica surface. Moreover, boric acid is known to form complexes with adenosine bases [93] that change the effective charge of the analytes and possibly may influence the interaction with surface as well.

First experiments were conducted with the BGE composed of 25 mM boric acid adjusted with sodium hydroxide to pH 9.5 and various amounts barium chloride were added. Table 1 shows electroosmotic mobilities with different content of the divalent modifier. Although promising results were achieved with 10 mM $BaCl_2$ as shown on Figure 16, the high barium chloride content contributed to increase in current (about 70 μA for at 25 kV). Further increase in amount of modifier which would be necessary for better separation of dcSAM diastereoisomers was not reasonable due to Joule heating produced known for deteriorating separations.

Table 1. Effect of $BaCl_2$ on electroosmotic mobility in 25 mM sodium borate pH 9.5.

mM $BaCl_2$	**electroosmotic mobility [× 10^{-9} m^2/Vs]**
0	47.73 ± 0.38
5	20.21 ± 0.63
10	15.05 ± 0.49
15	13.42 ± 0.56

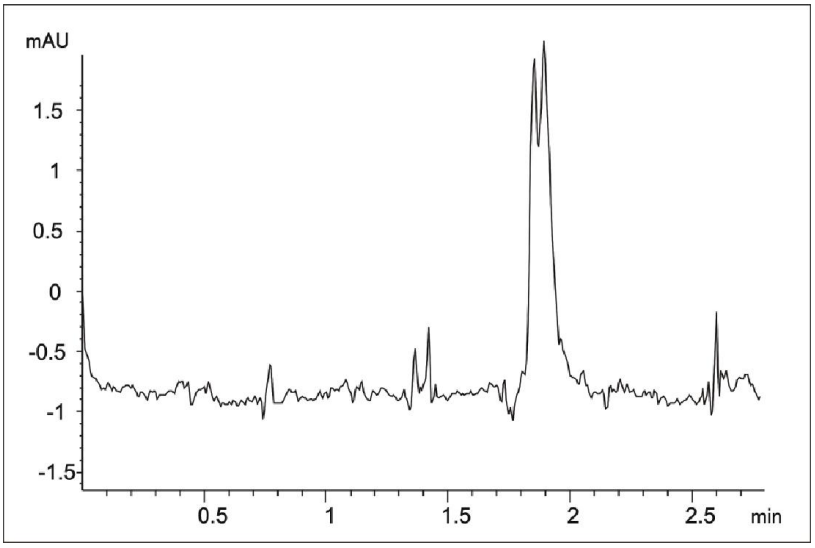


Figure 16. Separation of dcSAM diastereoisomers (dcSAM synthetic sample, 50 µg/ml) using 25 mM sodium borate pH 9.5 with 10 mM $BaCl_2$. Voltage +25 kV.

Next, possibilities of further reduction of electroosmotic mobility were investigated. The BGE based on 50 mM boric acid adjusted with barium hydroxide (20 mM) was tested; however, observed electroosmotic mobility of -12.1×10^{-9} m^2/Vs almost matched effective mobility of dcSAM (8.5×10^{-9} m^2/Vs in similar conditions shown on Figure 16). The EOF directed oppositely to dcSAM migration would unreasonably extend the analysis time.

Therefore, different ratios of barium and sodium borate buffers were mixed and tested. The expected effect of sodium cations was to counterbalance the influence of barium cations to reverse of migration, therefore by fine-tuning their ratio it would be possible to precisely manipulate with electroosmotic mobility.

Commonly observed effect in CE is a slight change in electroosmotic mobility over time explained by changes on the surface silica capillary due to gradual changes in ionization of silanol groups or adsorption of various compounds. The capillary could be imagined as a dynamic system that requires certain time to reach equilibrium to provide more or less stable electrokinetic properties. Therefore capillary rinsing is one of the important factors during a method development in order to provide repeatable runs. In the studied system of boric acid with sodium and barium hydroxide, it was found that for freshly pretreated capillary with 1M sodium hydroxide (5 min at 4.5 bar followed by rinsing with water) it takes long time till the surface reaches such equilibrium. Figure 17 shows dependence

of electroosmotic mobility on number of performed runs when a new capillary is started to use.

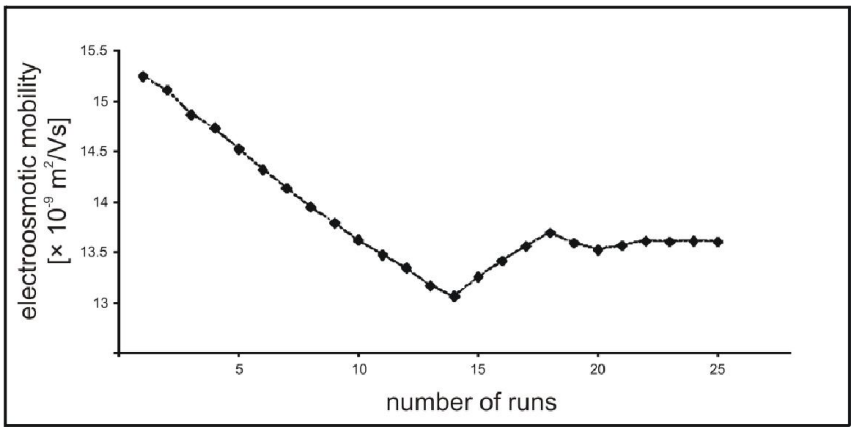


Figure 17. Dependence of electroosmotic mobility on number of runs using 50 mM boric acid, 10 mM sodium hydroxide, 10 mM barium hydroxide (pH 9.4). New capillary was first treated with 1M sodium hydroxide (5 min at 4.5 bar followed by water), then with the BGE (1 min at 4.5 bar) before each run that took 7 minutes. Electrolyte in vials was changed every five analyses.

It was not considered as satisfactory for method development and then use of the method that the EOF was stable after 20 runs (meaning almost 3 hours after start of using a new capillary). Together with problems with clogging of the capillary assigned to formation of barium carbonate precipitate in the capillary caused by absorption of carbon dioxide from the atmosphere into alkaline BGE, a solution was found in treatment of capillary by 0.1 M hydrochloric acid (0.5 min) before each run followed by the BGE (1 min).

Such rinsing step helped to remove barium carbonate; similarly CE vials contaminated with barium containing BGE had to be cleaned with hydrochloric acid in order to reuse them. The capillary surface was each time brought out from its way to equilibrium by the acidic treatment, therefore the memory effects were reduced as shown by intraday repeatability of electroosmotic mobility in the same BGE as 0.84 % RSD (n = 7). Interestingly the electroosmotic mobility changed from 13.6×10^{-9} m^2/Vs "equilibrated" state (considering last 5 runs shown in Figure 17) to 9.1×10^{-9} m^2/Vs when the capillary was rinsed with hydrochloric acid before each run.

Table 2 shows results obtained with several BGEs differing in sodium and barium content, amount of boric acid was kept at 50 mM. Along with measurements of the EOF, synthetic dcSAM standard was injected. Best results regarding resolution of dcSAM diastereisomers was achieved in the BGE composed of 50 mM boric acid, 10 mM sodium hydroxide, 10 mM sodium hydroxide (pH 9.4), Figure 18 shows electropherogram of the separation. Further improvement of the separation would be possible by change of the sodium/barium ratio in favour of barium; however dcSAM migration time would then have exceeded 10 minutes which was considered as a reasonable limit of the analysis.

Table 2. Electroosmotic mobilities observed in BGEs composed of 50 mM boric acid and different amounts of sodium and barium hydroxide. pH of the electrolytes was not measured.

mM Na^+	**mM Ba^{2+}**	**electroosmotic mobility [$\times 10^{-9}$ m^2/Vs]**
0	20	-12.14 ± 0.16
10	20	9.13 ± 0.12
10	10	9.67 ± 0.08
10	7.5	11.78 ± 0.09
20	10	13.59 ± 0.02
15	5.0	14.82 ± 0.11
20	2.5	16.75 ± 0.15
25	0	44.61 ± 0.21

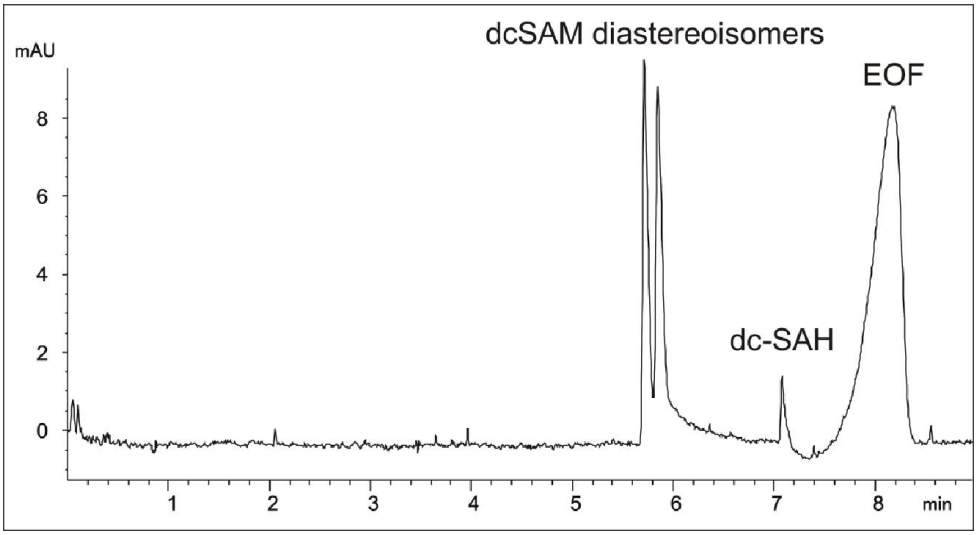


Figure 18. Analysis of dcSAM synthetic standard (120 μg/ml) using the BGE composed of 50 mM boric acid, 10 mM sodium hydroxide, 10 mM barium hydroxide (pH 9.4). Voltage +20 kV, detection at 210 nm, acrylamide as the EOF marker.

Identity of dcSAM diastereisomers was not resolved due to unavailability of corresponding standards. A newly present peak migrating between dcSAM diastereosiomers and the EOF marker was expected to be one its degradation product, decarboxylated *S*-adenosylhomocysteine (dcSAH), because of methyl-donor character of dcSAM. This was confirmed by reacting of dcSAM in ammonium hydroxide at 50 °C for two days and analyzing the product – dcSAM was quantitatively converted into peak migrating in 7th minute.

Parameters as repeatability of migration times, resolution, and limit of detection and quantitation of the developed method are summarized in Table 3 and 4.

Table 3. Intraday (n = 7) and interday (n = 3) repeatability of the method.

	t_{EOF} (min)	t_1 (min)	t_2 (min)	R_S
intraday				
average	8.22	5.76	5.90	1.35
RSD (%)	0.84	0.81	0.83	2.69
interday				
average	8.41	5.86	6.00	1.39
RSD (%)	3.12	2.57	2.59	3.85

Table 4. Detection limits of the method calculated by direct signal method (IUPAC recommendation).

	1. peak	**2. peak**
LOD (μg/ml)	17.9	17.5
LOQ (μg/ml)	25.5	25.1

4.1.8 Analysis of *S*-adenosylmethionine sample using barium borate

The developed method utilizing the BGE based on sodium-barium borate was applied on analysis of SAM sample. In order to prevent precipitation of the injected sample containing *p*-toluenesulfonate as counterion with barium borate based BGE, the sample was first treated with solid barium carbonate in an eppendorf vial. The suspension was centrifuged at 4500 rpm for 5 min and supernatant taken was analysis. Neutralization of the sample has to be noted as well, changing its pH from approximately 1.5 to about 6 after treatment with barium carbonate which improved repeatability of migration times.

Figure 19 shows analysis of SAM sample at concentration of 40 mg/ml using *N*-methylpyridinium iodine (0.3 mg/ml) as internal standard. Different migration time of dcSAM diastereoisomers could be attributed to different pH and ionic strength of the sample compared to analysis of only dcSAM synthetic standard. Content of dcSAM diastereoisomers in the sample was determined to be 0.114 ± 0.003 % for the first peak and 0.216 ± 0.005 % for the second peak. When SAM was introduced an opposite polarity was applied, it was not possible to obtain a signal of SAM within 20 minutes, but it was then

washed out from capillary by pressure. That suggests that under conditions of the method, the SAM migrates in the opposite direction than dcSAM diastereoisomers. Thus the main component does not interfere in determination of its degradation products which allows injecting even higher concentration the sample when lower amounts of impurities could be present.

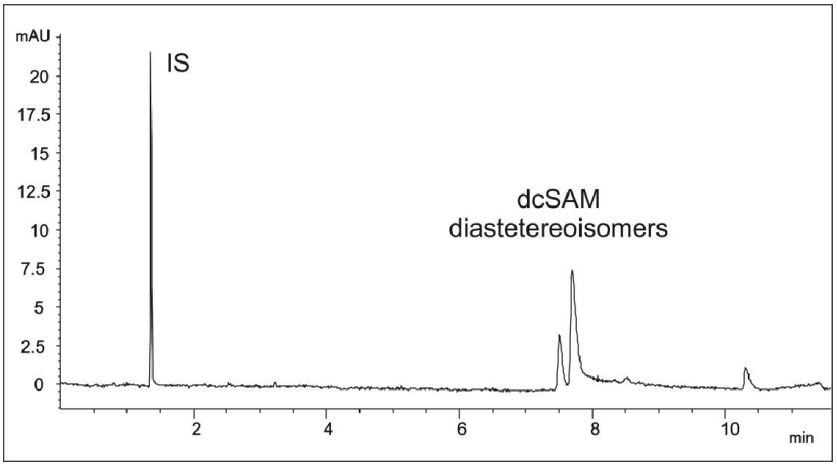


Figure 19. Analysis of SAM sample at concentration 40 mg/ml. *N*-methylpyridinium iodine (0.3 mg/ml) was used as an internal standard (IS).

4.1.9 Conclusion

In this part, several surface modifications of silica capillary surface were examined for separation of dcSAM diastereisomers from a pharmaceutical SAM sample. Such cationic compounds show significant tailing when encountering silanol groups at higher pH. Therefore capillary coating preventing such interaction has to be introduced.

Dynamic modification with cationic polymer (PVPy-Me), M7C4I, and covalent modification with polyacrylamide provided less satisfactory results than the BGE based on barium borate. Alteration of barium and sodium hydroxide content in the BGE allowed to tune electroosmotic mobility to desired value.

By adjusting experimental conditions, separation of diastereoisomers of dcSAM with resolution of 1.35 was achived in 10 minutes using 50 mM boric acid, 10 mM sodium hydroxide, 10 mM barium hydroxide (pH 9.4). The developed method could be utilized for impurity control in production monitoring of SAM. LOD of 17 μM and LOQ of 25 μM allows determination of content less than 0.1% in 40 mg/ml sample.

4.2 Enantioseparation of tartaric acid

Results shown in the section have been published in the following article:

- R. Knob, J. Petr, J. Ševčík, V. Maier, *Enantioseparation of tartaric acid by ligand-exchange capillary electrophoresis using contactless conductivity detection*. Journal of Separation Science 36, 2013, 3426-3431.

4.2.1 Tartaric acid

Tartaric acid is dicarboxylic acid possessing two hydroxy groups, its structure is shown on Figure 20. Tartaric acid is a chiral compound, it has two chiral centers located at the carbon atom linked with hydroxy and carboxylic functional groups. Along with its two enantiomers, mesotartaric acid exists and is a diastereoisomer to both of them; its structure is shown on Figure 20 and its analysis was not part of this study.

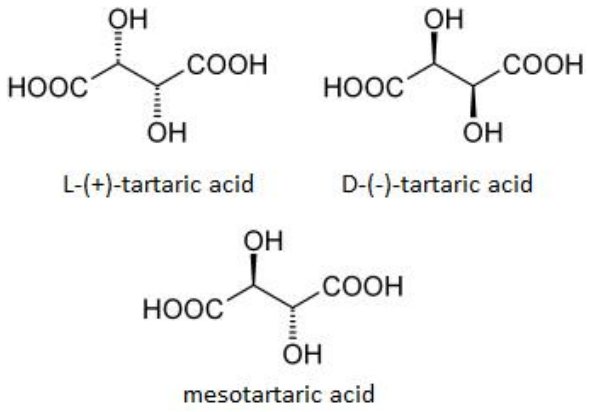


Figure 20. Structure of L- and D-tartaric acid and mesotartaric acid.

In nature, L-tartaric acid is the mostly occuring, it is distributed in plants and part of beverages as juices, wine, sake, etc. Oppositely, D-tartaric acid is much rare to find, some of *cis*-epoxysuccinate hydrolase-containing bacteria are able to produce it [94]; it also found use in pharmaceutical industry as a chiral intermediate.

D-tartaric scarcely occurs in nature, it can be considered as a marker of a bacterial contamination of beverages. The enantiomeric purity of tartaric acid used as a food additive

is required for safety and quality control of the products. Similarly, enantiomers of tartaric acid declared as a counterion of several pharmaceutical substances has to be controlled due to their potential adverse physiological effects.

4.2.2 Chiral separations

An important field of capillary electrophoresis lies in the separation of enantiomers of chiral compounds. Such analytes possess the same physicochemical properties and could be resolved only by interaction with another chiral compound which is called a chiral selector. The resulting diastereoisomeric complexes may differ in their stability and mobility, thus separation could be obtained. A wide variety of chiral selectors have been employed for resolving of enantiomers: cyclodextrins, macrocyclic antibiotics, or complexes of metal ion with chiral ligands belong among the most commonly used substances [95].

4.2.2.1 Chiral separations based on ligand exchange

Ligand exchange separation mechanism belongs to commonly used approaches for separation of enantiomers with complex-forming properties. It is based on interaction between the separated enantiomers and metal-ligand complex. Metastable diastereosiomeric complexes are formed differing in their properties like mobility or stability constant. Copper(II), zinc(II), and nickel(II) belong to the most frequently used metal ions. Analytes with suitable complex-forming properties could be separated, e.g. hydroxy acids, amino acids, amino alcohols, or diamines [96,97].

CE has been utilized in enantioseparation of tartaric acid by a group of Kodama who used chiral selectors based on copper(II) and nickel(II) with D-quinic acid [98,99] or (1*R*,2*R*)-(-)-1,2-diaminocyclohexane [100]. It was possible to change the migration order of the enantiomers, when concentration of D-quinic acid was lower than 40 mM D-tartaric acid was migrating first, with 80 mM and higher concentration of D-quinic acid the migration order was opposite. Authors applied developed methods for analysis of soft drinks, grape juice, sakes, and candies, however, no presence of D-tartaric acid above the detection limit was found.

4.2.3 Experimental conditions

Copper(II) chloride, *trans*-4-hydroxy-L-proline (L-OH-PRO), *cis*-4-hydroxy-D-proline, ε-aminocaproic (EACA), bis-tris, β-alanine, hydrochloric acid, D-tartaric acid, L-tartaric acid, γ-methacryloxypropyltrimethoxysilane (γ-MAPS), acrylamide, tetramethylethylenediamine (TEMED), potassium peroxodisulfate, and ethanol were obtained from Sigma-Aldrich (St. Loius, MO, USA). All chemicals were of analytical grade. Deionized water (Millipore, MO, USA) was used.

The analyses were performed by an HP^{3D} CE equipped with with the contactless conductivity detector of Gaš *et al.* [101] construction. The signal (0–1 V) from the CCD electronics was processed by the Agilent 35900E dual channel A/D converter (Agilent Technologies). Fused-silica capillaries (Polymicro Technologies Inc., Phoenix, AZ, USA, 75 μm ID, 60 cm total length, effective length 45 cm). Between runs the capillary was washed with the BGE for 5 minutes. Temperature of the capillary cassette was set to 25 ºC and separation voltage was -20 kV. Injection of samples was performed by application of pressure of 50 mbar for 5 seconds.

4.2.3.1 Covalent capillary modification by polyacrylamide

Modification of the capillary surface was considered due to anionic character of tartaric acid and expected EOF magnitude in an uncoated capillary in slightly acidic BGE that would be directed against the migration of the analyte. This would result in unreasonable long analysis time.

From the number of possible modifications leading into decrease of the EOF magnitude a covalent modification with polyacrylamide was chosen because of following reasons: (i) to suppress the EOF generation and allow tartaric acid migrate mostly according to its own effective mobility, (ii) to avoid introduction of any compound into the BGE that would interact with the capillary surface but it would be also involved in complex-formation equilibria for ligand-exchange separation, (iii) to reduce a risk of adsorption of matrix constituents of analyzed samples on the capillary surface that would induce a change in migration times of analytes between runs.

The surface modification was carried on according to previously published procedure with several modifications [102]. Capillary was first pretreated, starting with rinsing with 1 M sodium hydroxide for 3 hours, then with deionized water for 1 hour followed by 1 M nitric acid for 30 minutes and finishing with water.

Afterwards, capillary was washed with absolute ethanol followed by 20 % (v/v) solution of γ-MAPS in non-aqueous ethanol for 3 hours. Then capillary was washed with water for 30 minutes.

Next, capillary was continuously flushed with solution of 4% (w/v) acrylamide in freshly boiled and cooled (in order to remove carbon dioxide) deionized water with addition the initiator system composed of 0.1 % (w/v) potassium peroxodisulfate and 0.1 % (v/v) tetramethylethylenediamine (TEMED) for 12 hours. Finally, the capillary was rinsed with deionized water for 30 minutes.

Potassium peroxodisulfate acts as initiator which produces free radicals in presence of TEMED as a catalyst [102]. Unlike to commonly produced gels for polyacrylamide gel electrophoresis [103], a bifunctional acrylamide (*N,N*'-methylene-bisacrylamide) is not present in the polymerization mixture, therefore only linear polyacrylamide is produced. The polymerization reaction takes place in whole lumen of the capillary, however, the forming polymer is only attached at the methacrylate-functionalized capillary wall. Polymer chains unbound to the capillary are then washed away; in case of modification of longer capillaries it is necessary to provide enough time to remove the viscous solution. When ratios of the polymerization mixture constituents are not kept, polyacrylamide with high molecular weight (long chain) could be produced and capillary clogging may occur. Also temperature should be considered as it has substantial effect on polymerization kinetics.

4.2.4 Optimization of separation conditions

Based on previously reported separation of tartaric acid [98,99] and other hydroxy acids [104,105], chiral selector based on copper(II) chloride and L-OH-PRO was selected. For separation of organic acids, slightly acidic BGEs are often used providing enough selectivity and sufficient effective mobility regarding use of polyacrylamide coated capillary with suppressed EOF.

A set of compounds with different acidobasic properties (ß-alanine (pK_a 3.42, 10.24; values were obtained from Peakmaster software [106]), EACA (4.74), L-histidine (2.01, 6.04, 9.33), and bistris (6.40) was considered as the BGE counterion providing proper buffering capacity. It has to be noted that such compounds also form complexes with copper(II), however, an influence of their concentration was not studied separately regarding the resolution of tartaric acid enantiomers. Several BGEs composed of 5 mM $CuCl_2$, 10 mM L-OH-PRO and 50 mM of mentioned counterions were tested and compared regarding peak symmetry, CCD response, and resolution. Using ß-alanine, no enantioseparation was observed. Best results were achieved with EACA.

Interestingly, change in migration order was observed for BGEs containing EACA and bistris compared to presence of L-histidine. This was further studied using D-histidine as counterion as well as D-OH-PRO as the ligand. Results are summarized in Table 5. D-OH-PRO switches the migration order for EACA and bistris based BGEs, however, when D- or L-histidine is used, the migration order is not depending on the enantiomer of 4-hydroxyproline. This could be explained by different stability constants of the copper(II) and histidine complexes and also the kinetics involved. As D-tartaric acid is expected to be present as the minor enantiomer, its migration position as the first is more favorable, therefore the BGE composed of EACA and L-OH-PRO was chosen for further optimization.

Table 5. Migration order of tartaric acid enantiomers depending upon composition of the BGE.

BGE counterion	ligand	first peak	second peak
EACA	L-OH-PRO	D	L
bistris	L-OH-PRO	D	L
L-histidine	L-OH-PRO	L	D
D-histidne	L-OH-PRO	D	L
EACA	D-OH-PRO	L	D
bistris	D-OH-PRO	L	D
L-histidine	D-OH-PRO	L	D
D-histidne	D-OH-PRO	D	L

Next, effect of EACA concentration and pH of the BGE was examined. Best results were provided with 100 mM EACA with pH of the BGE adjusted to pH 5.0 by hydrochloric acid. Although slightly better resolution was achieved, lower pH prolonged migration time of tartaric acid to unreasonable times (longer than 13 minutes for pH 4.5). BGE pH higher than 5 led to significant decrease of response of the CCD detector.

In following step concentration of copper(II) (keeping the same 1:2 ratio with L-OH-PRO) and L-OH-PRO (with different ratios to copper(II): 1:0, 1:1, 1:1.5, 1:2, 1:2.5, 1:3) was studied regarding the resolution of enantiomers. Results are shown on Figures 21 and 22. In the BGE composed of 7 mM copper(II) chloride without L-OH-PRO it was not possible to obtain a signal of tartaric acid within 40 minutes suggesting strong complexation reducing its effective mobility. Similar results regarding the optimal ratio of metal ion and ligand have been also observed in other studies [104,105].

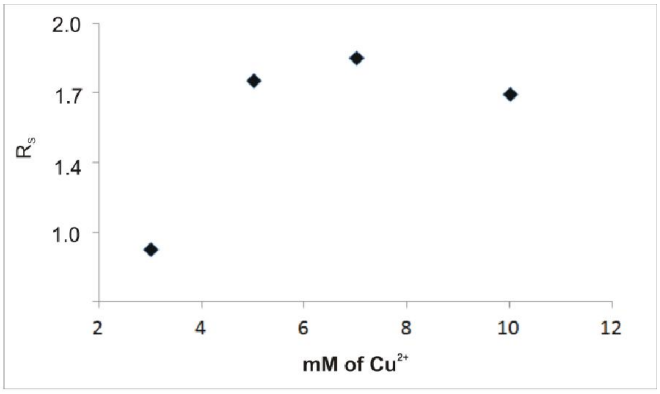


Figure 21. Dependence of resolution on concentration of Cu^{2+} in the BGE. L-OH-PRO was present at ratio 2 : 1 to Cu^{2+}, the BGE contained 100 mM EACA and its pH was adjusted to pH 5.0 by hydrochloric acid.

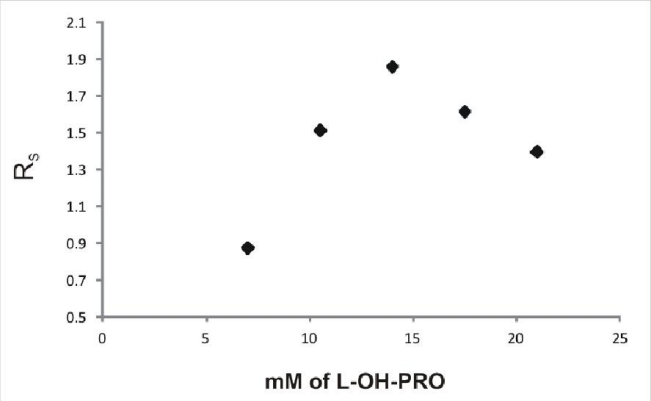


Figure 22. Dependence of resolution on concentration of L-OH-PRO. The BGE contained 7 mM Cu^{2+}, 100 mM EACA and its pH was adjusted to pH 5.0 by hydrochloric acid.

Optimal conditions were found with 7 mM copper(II) chloride, 14 mM L-OH-PRO, and 100 mM EACA pH 5.0 (adjusted by hydrochloric acid). Separation of a standard racemic mixture of tartaric acid enantiomers is shown on Figure 23.

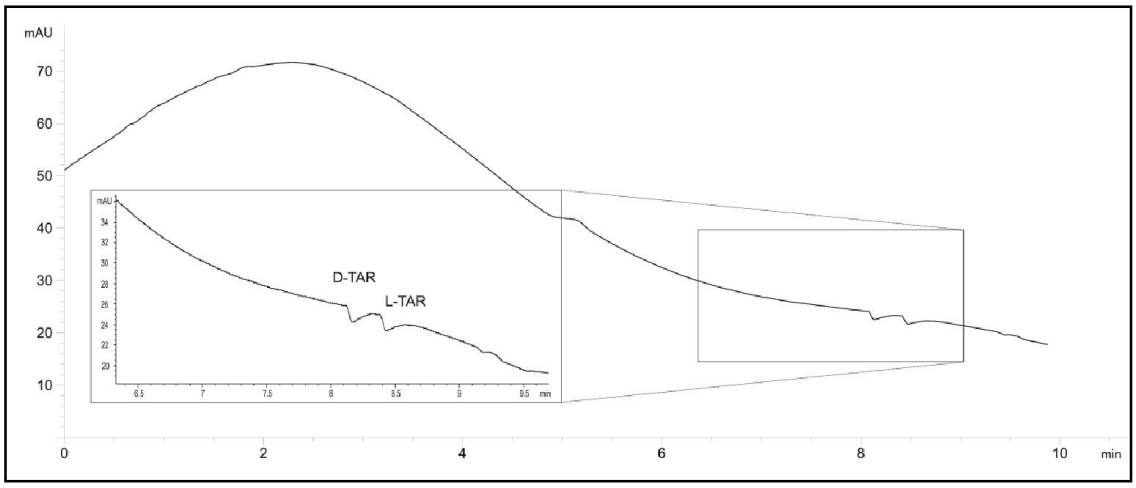


Figure 23. Separation of standard mixture of D,L-tartaric acid, concentration of each enantiomer was 50 µM. BGE: 7 mM $CuCl^2$, 14 mM L-OH-PRO, and 100 mM EACA pH 5.0.

Method parameters as repeatability of migration time, limits of detection and calibration equation are shown in Table 6.

Table 6. Intraday (n = 5) and interday (n = 3) repeatability of migration times, limits of detection and quantitation (calculated from calibration curve according to IUPAC recommendation using QCExpert 2.9), and calibration parameters (in range from 20 to 140 μM (six levels) with three repeats at each level).

	repeatability of migration time (% RSD)		LOD (μM)	LOQ (μM)	calibration equation	R^2
	intraday	interday				
D-tartarate	0.61	1.77	19.1	33.6	y = 0.1791x – 2.5280	0.9927
L-tartarate	0.62	1.89	20.6	35.9	y = 0.1821x – 2.6049	0.9916

4.2.5 Study of excess of L-tartaric acid

As L-tartaric acid is the mostly occurring enantiomer and D-tartaric acid scarcely exists in nature, low amounts of the minor enantiomer could be expected in samples where bacterial contamination is examined. In order to avoid of loss of resolution due to overloading, samples are diluted, but this is limited by the detection limit of the minor component. In case of other chiral selectors this could be overcome by increasing their concentration, however, in ligand-exchange the separation is based on interaction with single selective centre and further increase in concentration of chiral selector does not lead to improvement of resolution as was shown on Figure 22.

Figure 24 shows electropherograms of analyses of several mixtures of D- and L-tartaric acid differing in amount of L-tartaric acid, concentration of D-enantiomer was kept the same, close to the LOQ (40 μM). Till ratio 1D : 7L, baseline separation of the enantiomers is maintained and quantification fits the calibration curves. For ratio 1D : 10L the error of quantification of D-enantiomer was 17 % and for ratio 1D : 13L it was 28%. Dependence of the ratio on resolution is shown on Figure 25.

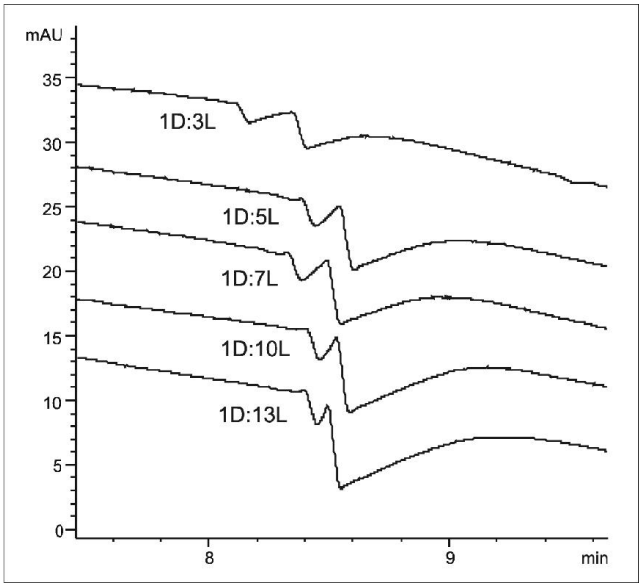


Figure 24. Separation of D,L-tartaric acid at different ratios. Concentration of the D-enantiomer was kept the same (40 μM), concentration of the L-enantiomer was increased in-the corresponding ratio.

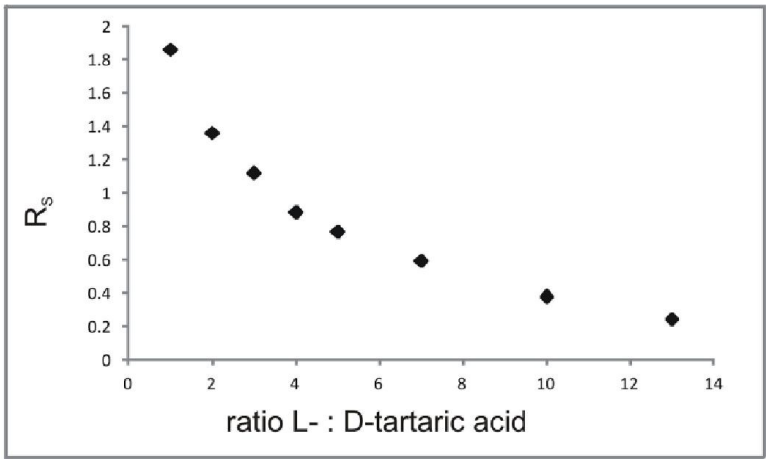


Figure 25. Dependence of resolution on ratio of L- and D-tartaric acid. Conditions the same as on Figure 23.

4.2.6 Sample analysis

The developed method was applied on analysis of beverage samples as wine, grape juice, and pharmaceuticals where tartaric acid is present as a counter-ion to the active substance. Figure 26 shows electropherograms of analysis of a white wine sample after 100 times dilution and the same sample spiked with D,L-tartaric acid with an increase

of the final concentration of 47 µM. There were no interferences found in the position of the two enantiomers in the samples.

The content of L-tartaric acid in white wine was found as 1.20 ± 0.09 g/l, in red wine 1.9 ± 0.1 g/l and in grape juice 5.3 ± 0.4 g/l. D-tartaric acid was not found in any sample proving no bacterial contamination. The recovery was 104 ± 4%.

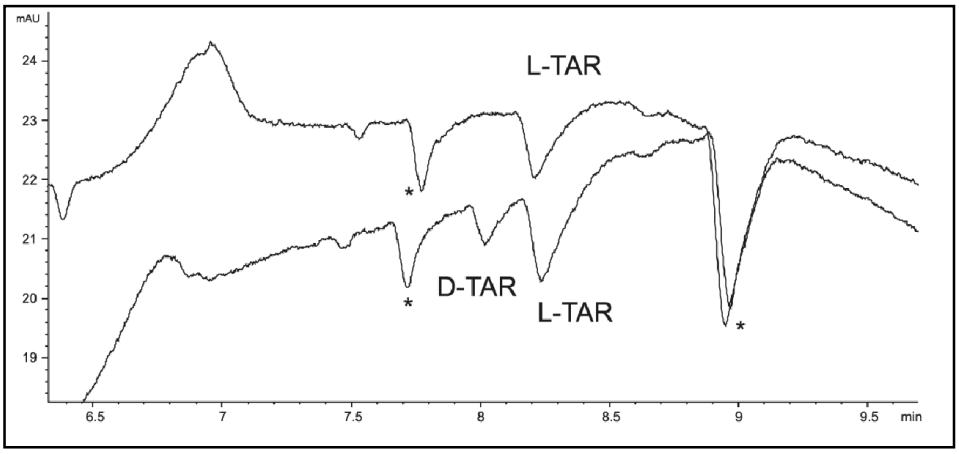


Figure 26. Analysis of 100 times diluted white wine sample (upper trace) and the sample spiked with D,L-tartaric acid (lower trace).

Purity of pharmaceutical substances was assessed in formulations of metoprolol tartrate and zolpidem tartrate. Analysis of 0.1 mg/ml metoprolol is shown on Figure 27. Only L-tartrate was found in the samples, content found in the 0.1 mg/ml samples was as follows: 34 ± 2 µg/ml in metoprolol (corresponds to 95.5 % of the theoretical content assuming 100% purity) and 31 ± 2 µg/ml in zolpidem (corresponds to 94.6 % of the theoretical content).

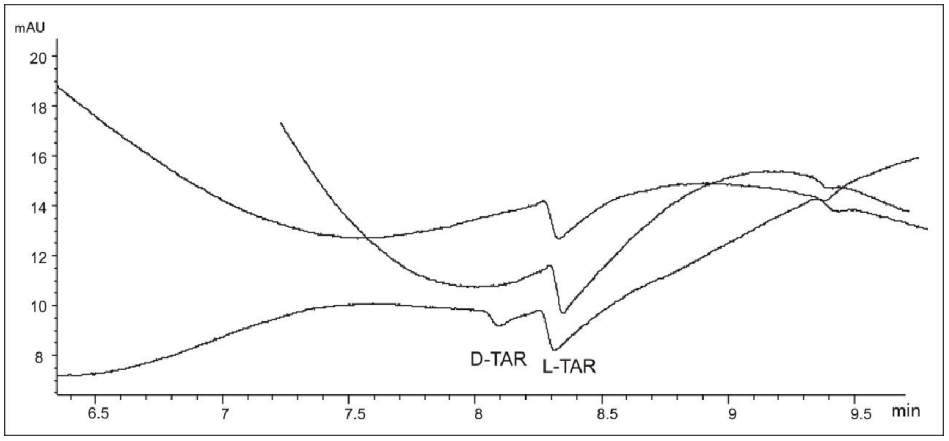


Figure 27. Analysis of 0.1 mM metoprolol tartarate (upper trace), the sample spiked with 30 µM L-tartaric acid (middle trace), and the sample spiked with 30 µM D-tartaric acid (lower trace).

4.2.7 Conclusion

In conclusion, method for separation of tartaric acid enantiomers was developed. The optimal conditions regarding resolution and analysis time were found in the BGE composed of 7 mM $CuCl_2$, 14 mM *trans*-4-hydroxy-L-proline, and 100 mM ε-aminocaproic acid with pH adjusted by hydrochloric acid. The method is based on contactless conductivity detection, a low cost alternative to UV detection, and in this case CCD was utilized in ligand-exhange based chiral separation for the first time. Polyacrylamide coated capillary was used for suppression of the EOF and elimination of adsorption of matrix constituents.

Several counter-ions were tested and the possibility of reversal of migration order of tartaric acid enantiomers was studied with use of both isomers of 4-hydroxyproline. As it is advantageous when the minor enantiomer migrates as the first, the BGE composed of *trans*-4-hydroxy-L-proline and ε-aminocaproic acid was used. The separation was achieved in 9 minutes with a resolution of 1.9. Decrease of resolution was studied for sample with various ratios of D- and L-tartaric acid; baseline separation was maintained till ratio 1D : 7L.

The method was applied on analysis of wine, grape juice, and pharmaceutical formulations, however, no D-tartaric acid was found.

4.3 Porous layer open tubular columns fabrication

Monolithic, or continuous porous stationary phases have attracted significant interest due to their porous properties allowing the use of high flow rates at low backpressures, relatively simple preparation in capillaries with small internal diameters, lack of frits and the ability to control both the physical and chemical properties of the material [107-110]. These properties make them very attractive for PLOT columns.

Among many approaches for fabrication of PLOT columns, very recently Collins *et al*. [111] have reported use of a device containing several circular arrays of UV light emitting diodes (LED) and the capillary is delivered through the centre of a UV chamber to provide equal intensity to all sides of the capillary thus ensuring an evenly distributed porous polymer without rotation of the capillary. A number of parameters is expected to have an impact on the resulting polymer allowing to tune the product.

4.3.1 Photoinitiation using LED oven

Light emitting diodes are semincondutor-based light source and found use in many areas of everyday life. With recent development in miniaturization and advances in their power output they attracted attention as low-cost light sources for detection systems in homemade [112] and modified commercial instruments [113] as well as in microfabricated devices [114]. LEDs now cover broad range of spectra ranging from infrared through visible to UV part of spectrum. However, deep-UV (lower than 300 nm) LEDs are still considerably expensive, they require cooling and their power output is limited.

4.3.1.1 Construction of LED oven

For construction of LED oven, cheap but still reasonably powerful LEDs in range of near-UV part of spectrum (below 400 nm) were considered. Based on specifications provided by manufacturers, two LEDs were chosen for comparison: 370 nm LED “MARL 260019” [115] and 385 nm LED “Lumex SSL-LXTO46UV1C” [116]. Their power output was briefly evaluated using OAI 306 UV Powermeter (Optical Associates, San Jose, CA, USA) equipped with a 365 nm probe by applying the maximal allowed current at voltage

recommended by the manufacturer (15 mA, 3.6 V and 20 mA and 3.3 V, respectively). The 370 nm LED provided output of 4.6 ± 0.1 mW/cm^2 and the 385 nm LED 13.6 ± 0.4 mW/cm^2 (n = 5). The 385 nm LED was chosen as it provided far better power output, even though its peak wavelength was farther than the wavelength at which the powermeter's probe operated.

A scheme of the LED oven consisting of 4 LEDs is shown on Figure 28. 18° beam angle was expected to require only 4 LEDs in order to cover the surface of about 360 µm O.D. capillary, the number of LEDs arranged in a circular array was found to be still simple to assemble as shown on Figure 29. Furthermore, PTFE tubing (0.5 mm ID, 1 mm OD) was inserted in the centre of the array to act as a guide for the capillary and also to disperse the light coming from LEDs to provide more homogeneous coverage. After soldering the LEDs into a parallel circuit, the device was placed near to a step-motor operated rubber holder. Rotation of the rubber wheels allowed to drag the capillary through the LED oven at given speed controlled by the step motor. The thickness of the polymer could be then controlled by variation of the movement speed and the intensity of the LEDs.

Figure 28. 385 nm LED "Lumex SSL-LXTO46UV1C" (left) and a scheme of the LED oven (right).

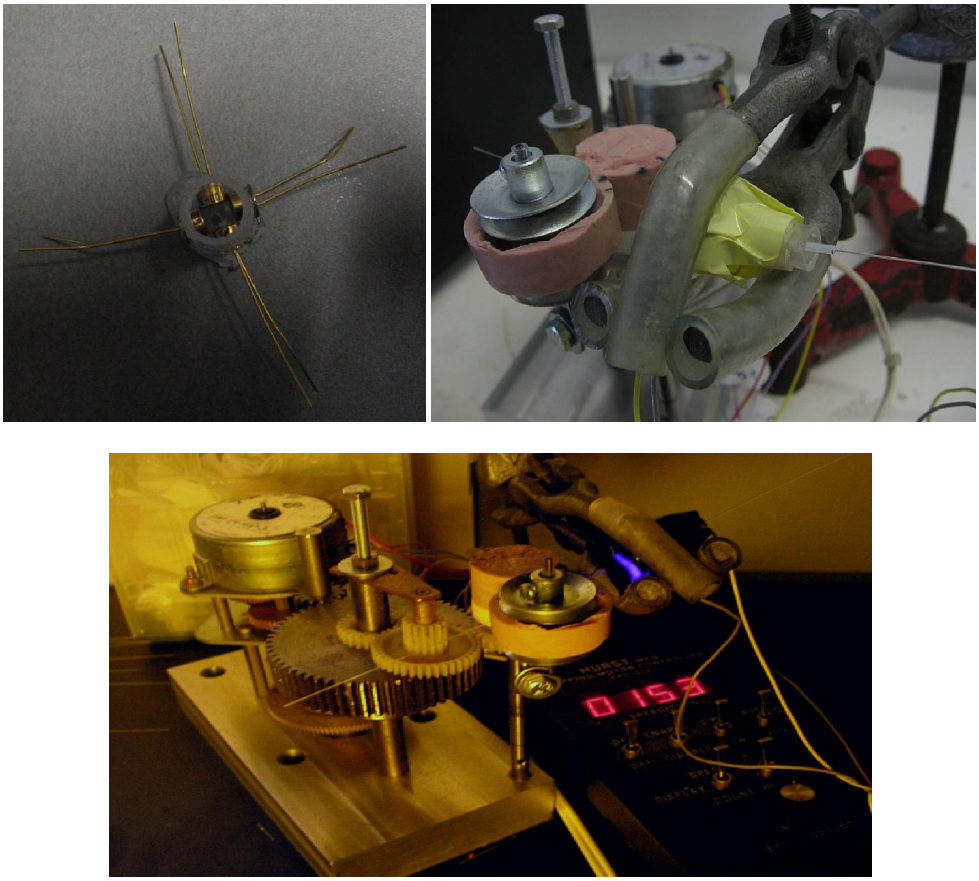


Figure 29. LED oven consisting of 4 LEDs in a plastic holder before soldering (left) and assembled LED oven near step-motor controlled rubber wheels for capillary delivery (right and bottom).

4.3.1.2 Selection of photoinitiator

For photoinitiated polymerization of monolithic stationary, initiators as 2,2-dimethoxy-2-phenylacetophenone [117] and dibenzoyl peroxide [118] are often used. However, these compounds are often irradiated by deep-UV lamps as they absorb light mostly below 300 nm and thus could not be efficiently used with LEDs emitting light at 385 nm.

Based on the information sheets provided by suppliers [119,120], bis(2,4,6-trimethylbenzoyl)-phenylphosphineoxide (BAPO, Irgacure 819) was considered as a suitable initiator for the purpose of the assembled LED oven. BAPO has been already used for preparation of a monolithic column for heterogeneous catalysis [121]. Structure of BAPO is shown of Figure 30 and its absorption spectra on Figure 31. It has to be noted that the absorption spectra is overlapping with the ambient and fluorescent lamp lighting

in the laboratory, the polymerization has to be done either in dark or in a “yellow room” with lighting absent of lower wavelenghts.

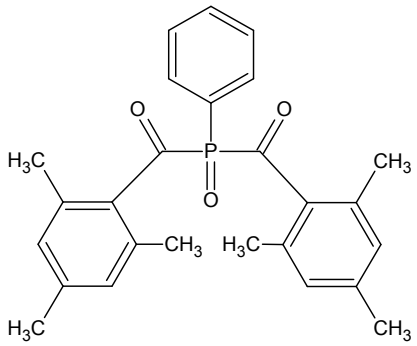


Figure 30. Structure of BAPO.

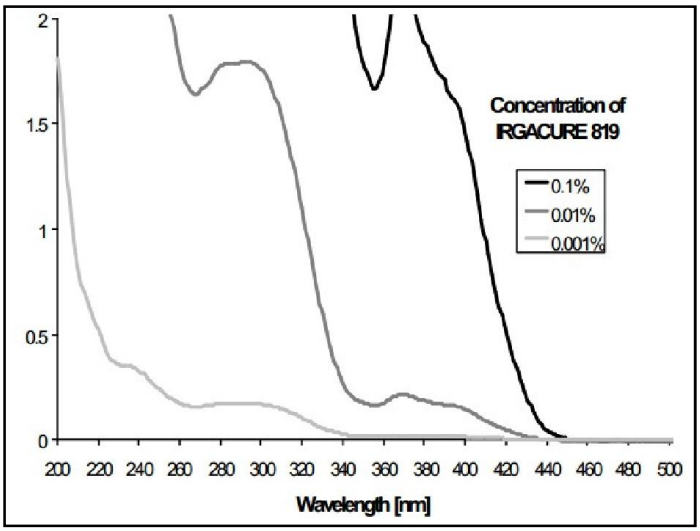


Figure 31. Absorption spectrum of BAPO (Irgacure 819), adapted from [122].

For the experiments, composition of the polymerization mixture was used the same as in previously in-house prepared monoliths which were tested for optimal flow-through properties and minimization of shrinkage after the polymerization [121]. The prepared monolith was a copolymer of ethylene dimethacrylate (EDMA) as a linking monomer and glycidyl methacrylate (GMA) as a functional monomer; the epoxy functional groups could be further modified by different reactions to a variety of functional groups depending upon application of the column. 1-dodecanol and cyclohexanol were used as porogenic solvents, the composition of the polymerization mixture was following: 0.4 g EDMA,

0.6 g GMA, 1.4 g 1-dodecanol, and 0.93 g cyclohexanol. The initial content of BAPO was 1 % (m/m) relative to monomers.

4.3.1.3 Characterization of columns by optical profilometry

In order to characterize the produced porous polymer and to assess the volume of the capillary lumen filled with the polymer, SEM is utilized in the most cases. However, due to limited accessibility, running costs, and time consuming sample preparation, a different option was searched for to provide fast and easy way for brief inspection of the produced capillary columns.

Optical profilometry was evaluated as a tool for quick assessment of the cross-section of the capillary lumen filled by the porous polymer which is essential parameter of successful formation of PLOT stationary phase. In comparison to SEM, optical profiler operates at ambient conditions, the instrument is immediately ready to use and does not require any specific sample treatment than positioning the capillary upright as shown on Figure 32.

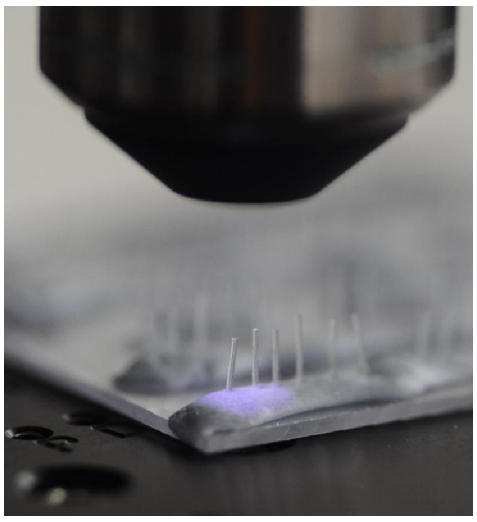


Figure 32. Capillary cuts positioned for optical profilometry measurement using Blu-Tack adhesive.

Wyko NT9100 Optical Profiler (Veeco, NY, USA) was used for assessment of the columns. The measured surface has to be perpendicular to the beam of the microscope, therefore response from an irregular surface of porous polymer is not obtained from every

part of the structure. Limitation was found in lateral resolution of about 1 µm (when using lens with 40x magnification) which limits its use mostly to thicker layer of polymer coating.

Usually only small part of the polymer is visualized on the record as illustrated on Figure 33 showing a comparison between bare fused silica capillary and capillary modified with a thin layer of polymer. Figure 34 and further figures illustrate several situations visualized by both optical profilometry and SEM. As Teflon-coated silica capillaries of 50 µm ID were used in all experiments, the size scale on profilometry images are not further shown as they correspond to scale shown on Figure 33.

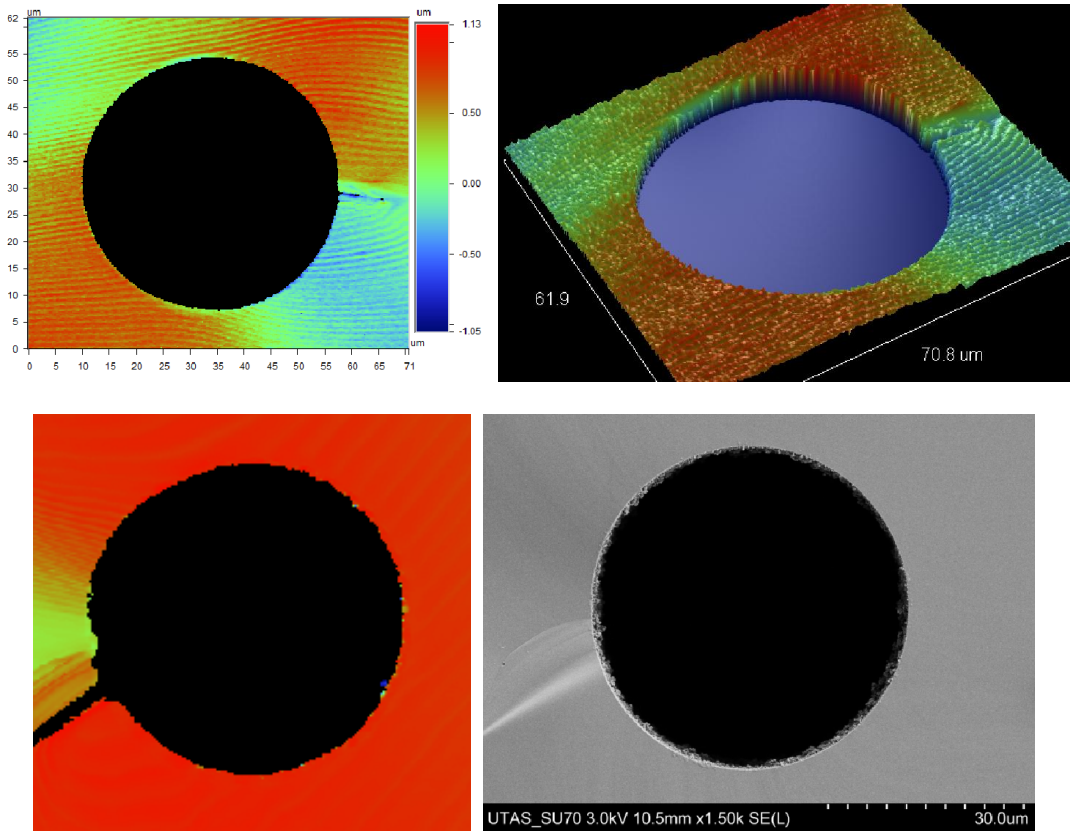


Figure 33. Comparison of optical profilometry visualization of unmodified silica capillary (top) with capillary modified with about 1 µm thick polymer coating and corresponding profilometry (left bottom) and SEM image (right bottom).

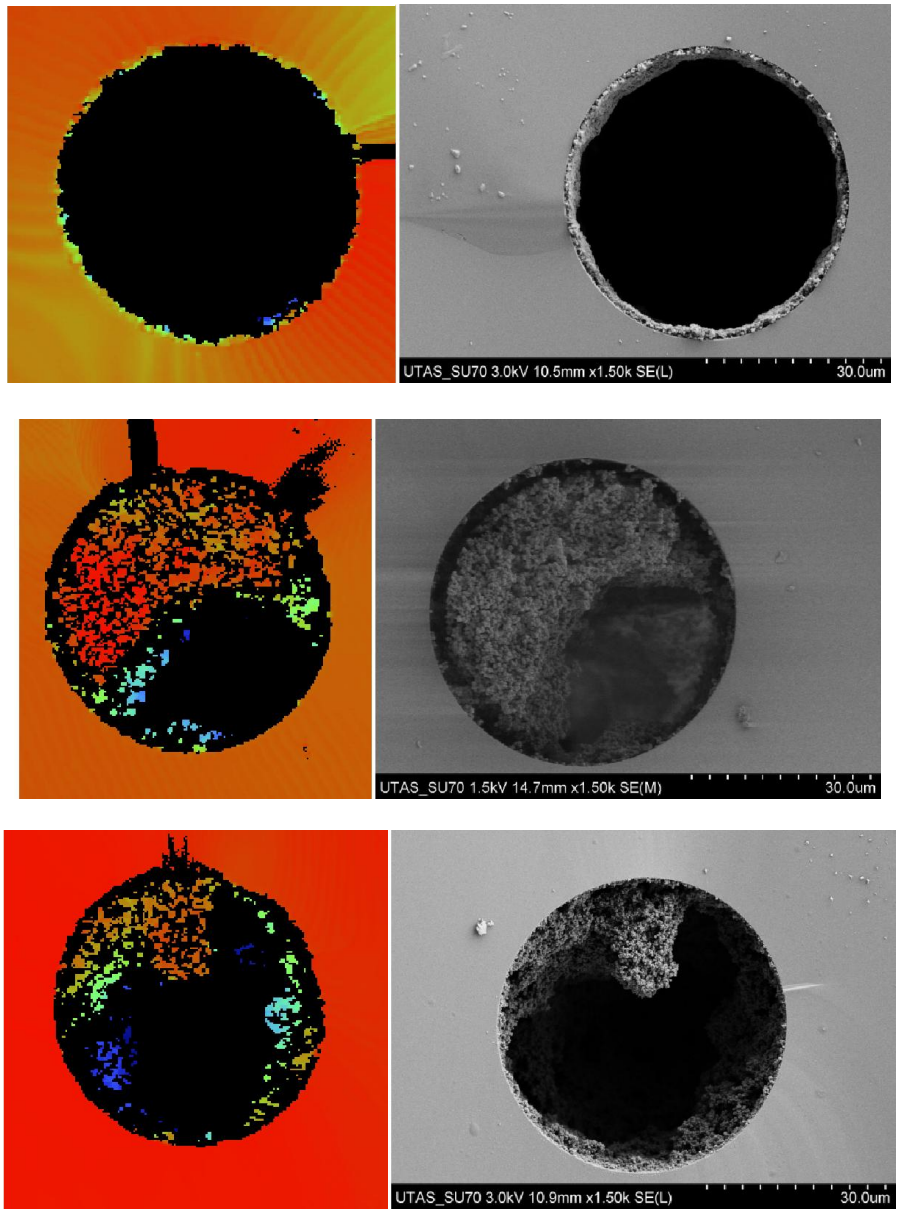


Figure 34. Comparison of three selected capillary cuts. Corresponding optical profilometry image on left and SEM image on right.

4.3.1.4 Polymerization using LED oven

For the first set of experiments, 1.9 mm/min delivery speed of the capillary was selected and three segments of the γ-MAPS treated (for modification process details, see section 4.2.3.1) Teflon-coated silica capillary were exposed using different current input on the LED oven: 5, 10, and 20 mA. The process resulted in all segments in formation

of the monolith in the whole volume of the capillary suggesting that the dosage exceeded the energy required to only partly polymerize the solution. The result is shown on Figure 35.

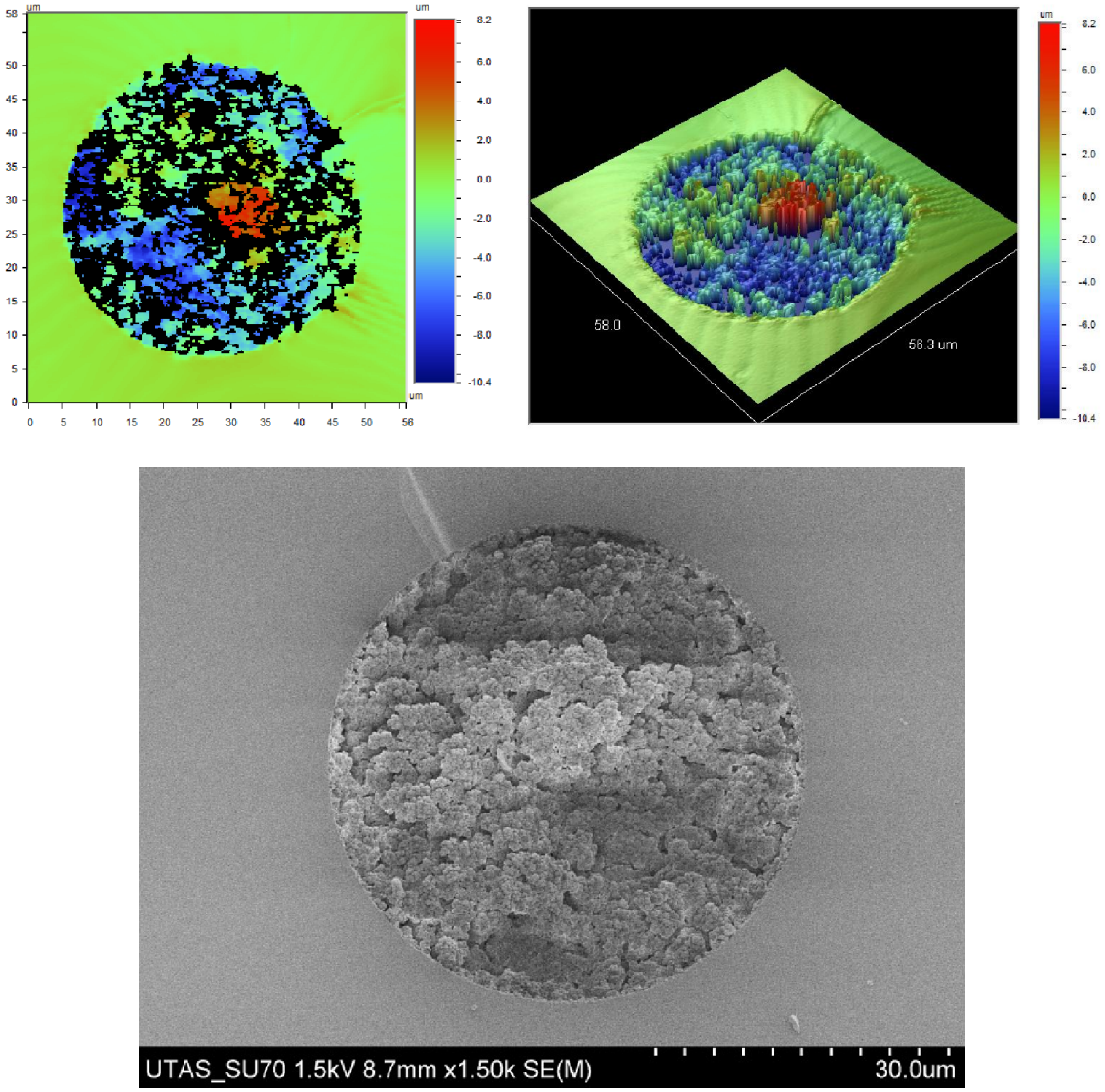


Figure 35. SEM and profilometry images in 2D and 3D view of the porous polymer filling whole column lumen produced with 1% BAPO.

When lower input than 5 mA was set on the LED oven, the device did not produce any light; therefore other options had to be considered. Next, the delivery speed of capillary was increased to 5.5 mm/min (maximum setting) and content of BAPO was reduced to 0.2 % (m/m) relative to monomers. Different composition of the polymerization mixture and faster speed resulted in decrease of the polymerization speed as confirmed on Figure 36. The result

shows formation of the porous polymer in majority of the capillary volume, however, the significant inhomogeneity of the distribution of porous polymer was observed.

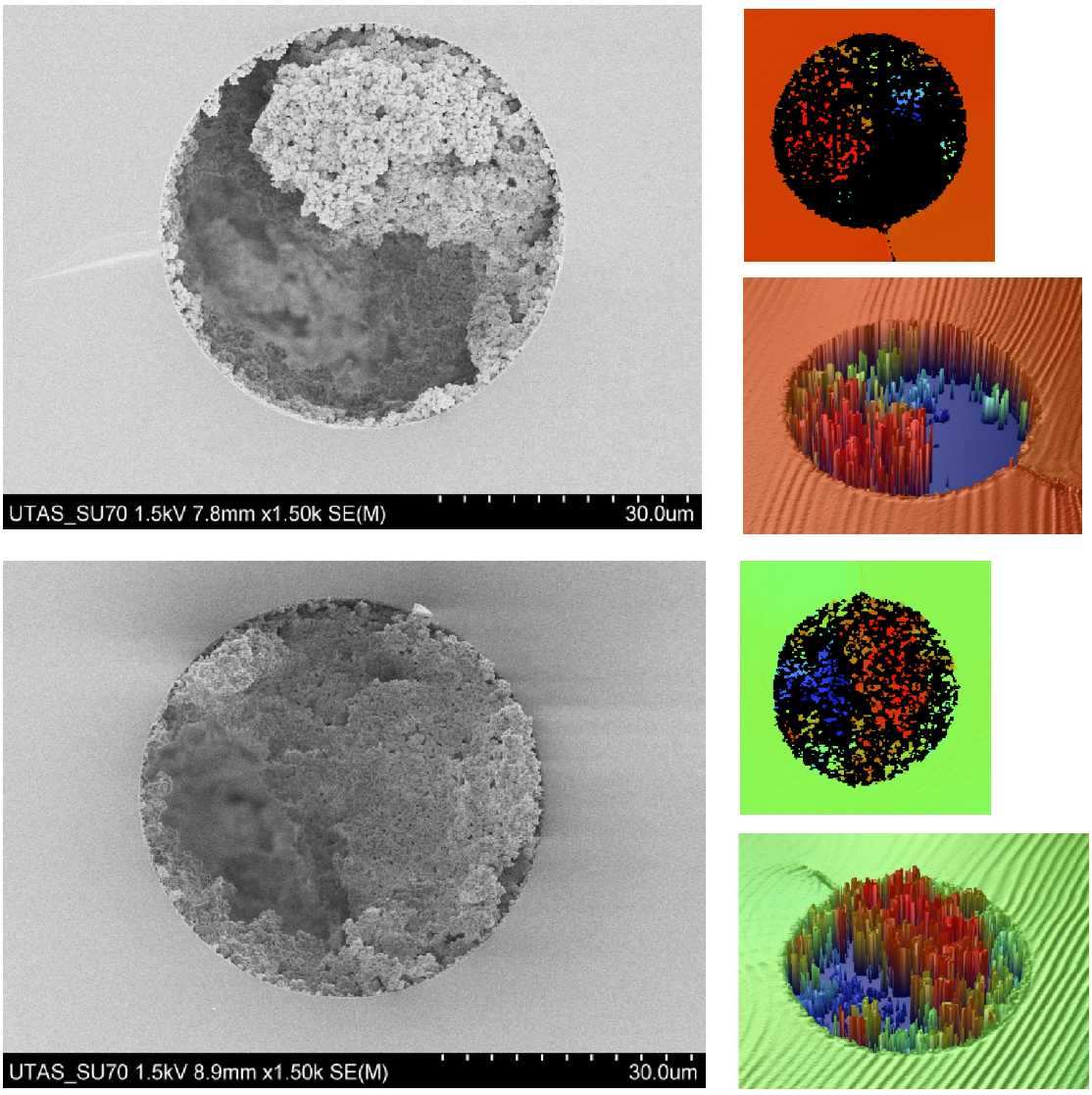


Figure 36. SEM (left) and optical profilometry (right) images in 2D and 3D view of produced column (two cuts of the capillary) with 0.2 % BAPO, 5.5 mm/s capillary speed and 5 mA LED oven input.

Further decrease of BAPO to 0.1% led to largely unrepeatable results possibly explained by increased sensitivity of the polymerization process to impurities and presence of absorbed oxygen over time. Therefore other options of influencing the polymerization kinetics were considered.

4.3.1.4.1 Polymerization inhibition by hydroquinone

Methacrylate-based monomers are often supplied with some content of an inhibitor to prevent deterioration by polymerization during storage. In case of EDMA and GMA 100 ppm of monomethyl ether hydroquinone is declared by manufacturer. Before use it is recommended to remove the inhibitor by beads containing inhibitor removers (provided by Sigma-Aldrich without further specification).

For the next experiment, GMA and EDMA were used without removal of monomethyl ether hydroquinone. Polymerization mixture containing the inhibitor, 0.2 % BAPO was polymerized in the capillary with speed of 5.5 mm/s with 5 mA input. The result is shown on Figure 37. The presence of inhibitor affected the polymerization kinetics by reducing the amount of polymer produced compared to the results obtained with the same setting on the LED oven (Figure 37).

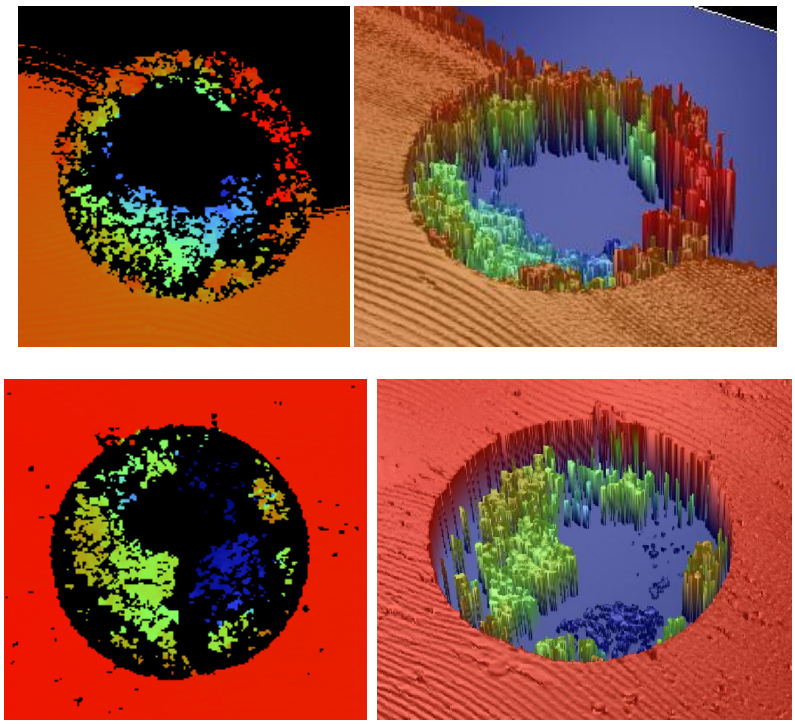


Figure 37. Profilometry images of produced column (4 different cuts) with polymerization mixture containing monomethyl ether hydroquinone.

In order to control the amount of the inhibitor and hence control the thickness of the polymer coating on the capillary wall, different content of hydroquinone in the polymerization was studied. Hydroquinone was selected for its availability and due to structural similarity identical effect was expected. Figure 38 shows SEM images obtained from 10 cm long columns from which 5 cm was passed through the LED oven and polymerized with 0.2 % BAPO and capillary with speed of 5.5 mm/s with 5 mA input to the LED oven.

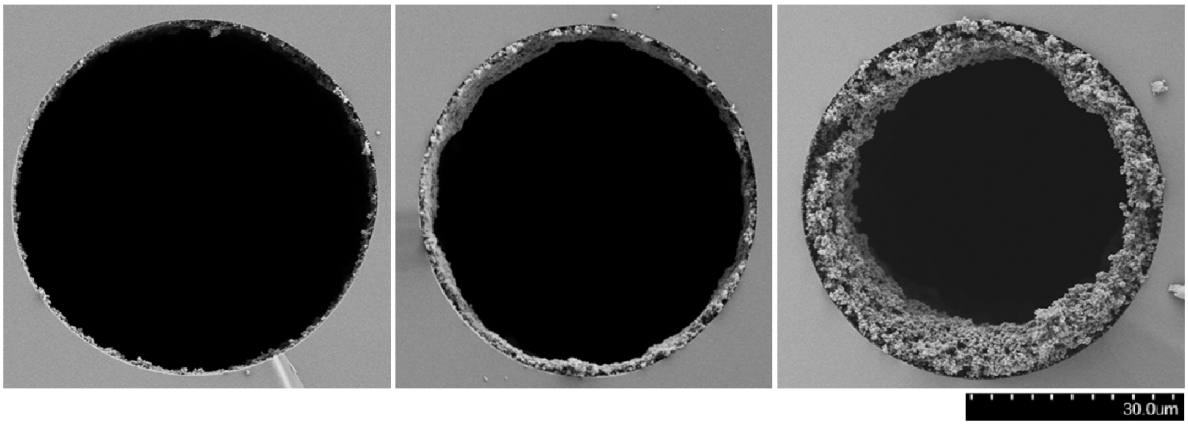


Figure 38. SEM images of columns produced with polymerization mixture containing hydroquinone at concentration 1 mg/g (left), 0.8 mg/g (middle), and 0.5 mg/g (right).

As it was successful to produce PLOT columns with different thickness of the layer (about 1 µm for 1 mg/g of hydroquinone, 3 µm for 0.8 mg/g, and 6-10 µm for 0.5 mg/g), the procedure was applied on capillaries 50 cm in length that could be next used in Agilent CE for capillary electrochromatography. However, when longer columns were delivered through the LED oven, inhomogeneity of the thickness distribution was observed over the length of the capillary as demonstrated on Figure 39 showing images from cuts at different places.

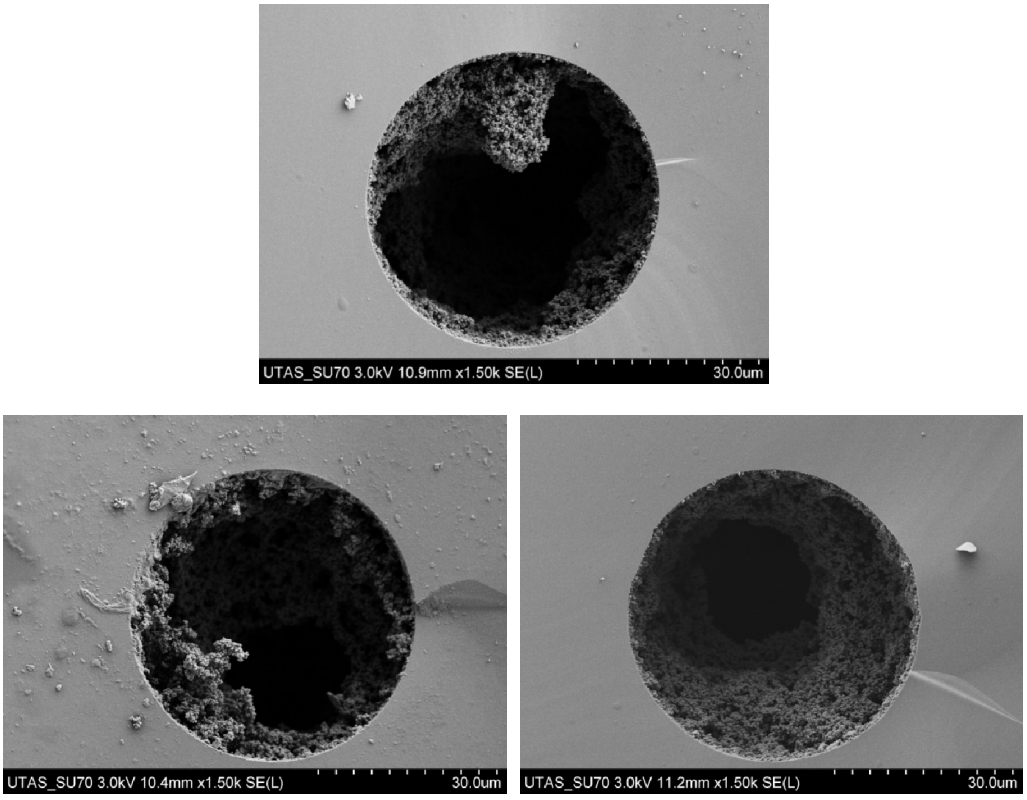


Figure 39. SEM images of different capillary cuts from a single column polymerized with 0.5 mg/g of hydroquinone.

The inhomogeneity could be explained by light propagation in the Teflon-coated silica capillary. It was observed that the more distant end of the capillary is mildly shining (in dark) when the other end is lit by the LED oven. Such effect was observed for even 1.5 m long capillary. Therefore when light is propagated over the capillary length during the entire process, different places are irradiated at different time before and after they pass through the LED oven. This could result in inhomogeneity as polymer particles formed in the centre of the capillary could attach to the polymer at the capillary wall, most probably from the point where the capillary was irradiated first.

4.3.1.4.2 Light attenuation by 4-nitroaniline

To suppress the described effect of light propagation in the capillary an addition of light-absorbing compound was considered. That would limit the amount the light penetrating the polymerization solution deeper inside the capillary and hence increase the chance of formation of the polymer only at the capillary wall.

At first, light attenuation of BAPO itself was examined. For evaluation of the amount of absorbed light Lambert-Beer law was taken into account:

$$A = \varepsilon . c . d \quad (6)$$

where A represents absorbance, ε decadic molar absorption coefficient, c concentration of the compound and d pathlength. Absorbance is defined as follows:

$$A = -log_{10}\left(\frac{I}{I_0}\right) \quad (7)$$

where I_0 represents initial light intensity, I light intensity of transmitted light. From the equations *(6)* and *(7)* the dependence of transmitted light intensity could be derived:

$$I = I_0 . 10^{-\varepsilon cd} \quad (8)$$

Using the equation *(8)* a dependence of the light attenuation (reflected as decrease of transmitted light) on the distance passed through a solution could be constructed. In the case of BAPO, an estimation of decadic molar absorption coefficient as 770 $dm^3.mol^{-1}.cm^{-1}$ at 385 nm was obtained from the data shown on spectrum on Figure 31.

The attenuation in scale of 50 μm (capillary ID) for solutions of BAPO at concentration 0.2 %, 1 % and 5 % is shown on Figure 40. The lowest concentration shows not significant decrease in light intensity, 1 % solution decreases light intensity by 93.75 % after passing through the capillary. 5 % solution is shown only for illustration as an estimation, such concentration would result in significant light attenuation, however, this amount is impractical for fast polymerization kinetics and initiator solubility as well.

It has to be noted that only one light source was considered – in the constructed LED oven there are 4 LEDs, therefore overlap of their light output is expected. For simplicity attenuation from only one light source is shown. Also any solvent effect on change in absorption coefficient was not considered as the data are supposed to provide only supportive information.

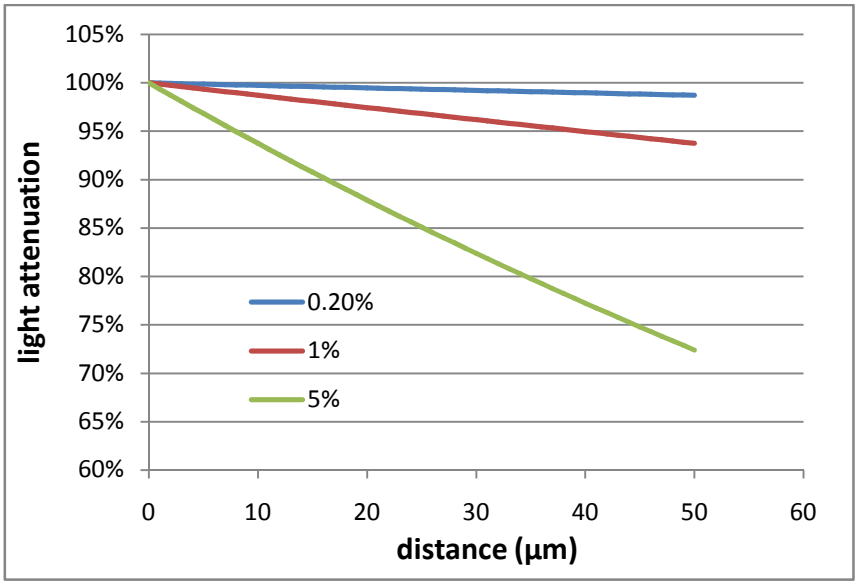


Figure 40. Light attenuation of BAPO solutions at different concentrations.

Insignificant light attenuation of BAPO therefore required introduction of another compound that would limit the amount of passed light deeper into capillary. Several yellow coloured compounds were considered: 4-nitroaniline, dinitrosalicylic acid, and martius yellow (2,4-dinitronaphthalen-1-ol). 4-Nitroaniline (4-NA) was selected for further experiments for good solubility in polymerization mixture, its absorption spectrum is shown on Figure 41.

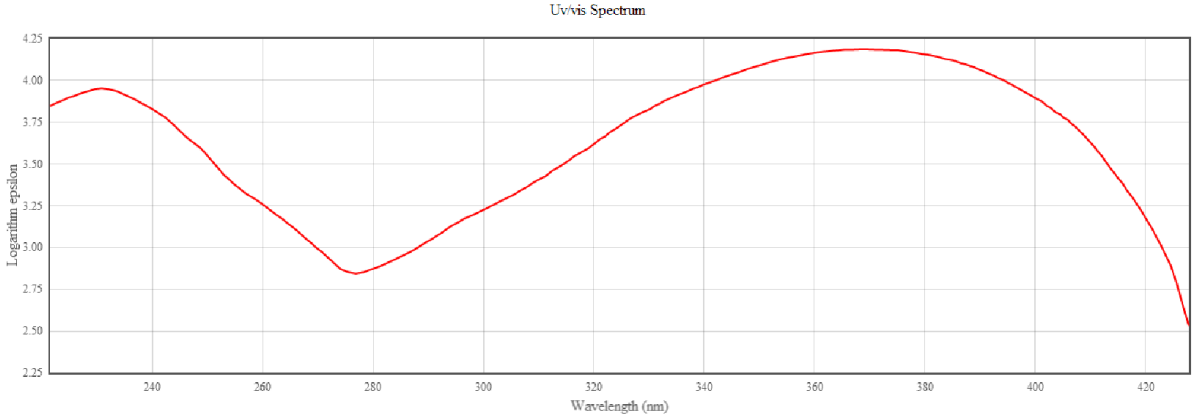


Figure 41. Absorption spectrum of 4-nitroanaline (obtained from [123]).

Addition of 4-NA to the polymerization mixture containing 1 % BAPO was first briefly evaluated at concentration range from 1 to 5 mg/g under 365 nm LED lamp. Whereas polymerization mixture without 4-nitroaniline was fully polymerized within about 2 minutes,

at concentration of 1 mg/g about half of the volume in a test tube was polymerized after 1 hour. For higher concentrations, only a slight change of viscosity was observed with no visible presence of polymer.

Light attenuation by 4-nitroaniline in dimensions of 50 µm ID capillary was calculated, according to Figure 41 decadic molar absorption coefficient of 4-NA was estimated as 14000 $dm^3.mol^{-1}.cm^{-1}$. Results for three different concentrations are shown on Figure 42.

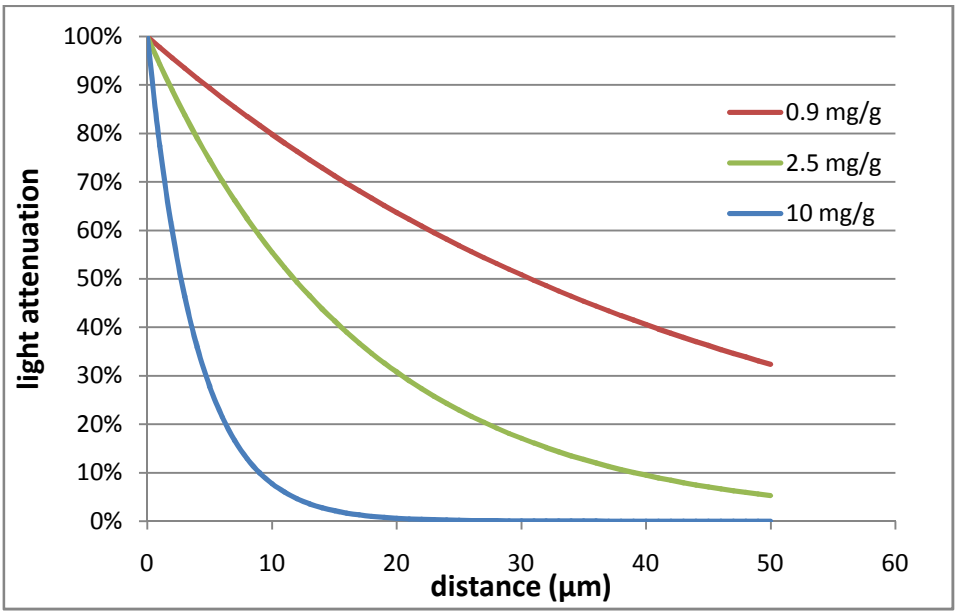
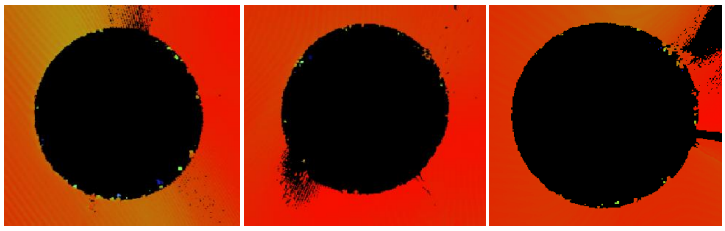
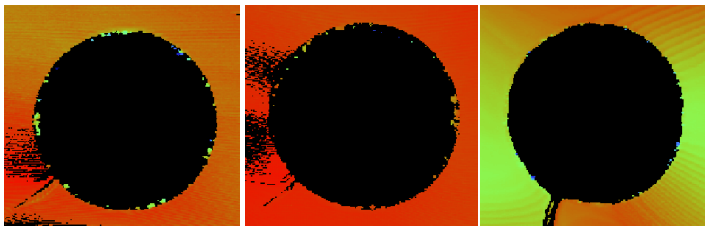
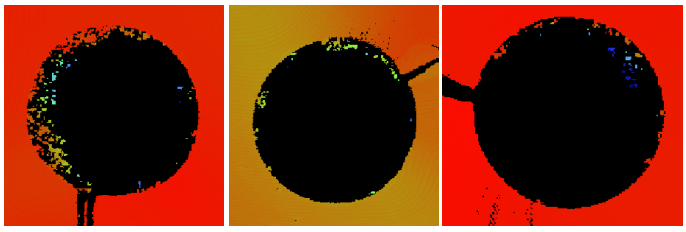
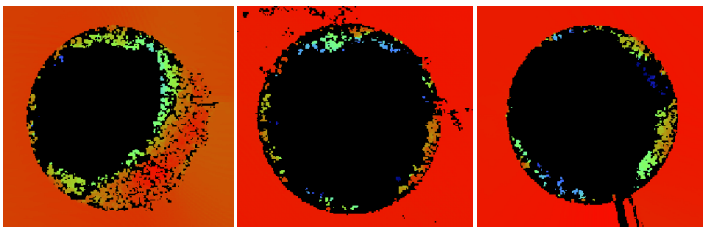
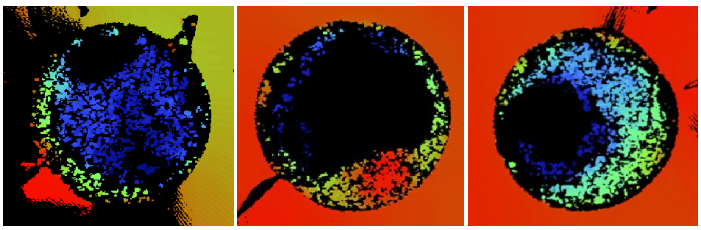
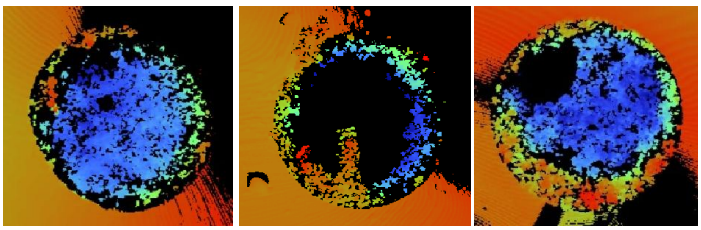


Figure 42. Light attenuation by 4-nitroaniline at several concentration levels.

Possibility of light attenuation by 4-NA was examined experimentally using polymerization mixture with varying content of BAPO and several settings of capillary speed and LED oven input. In Table 7 there are shown images of cuts from six produced PLOT columns. Cuts were obtained from several spots at the beginning and at the end of the column.

Table 7. Profilometry images of selected representative cuts of PLOT columns produced using polymerization mixture with various content of BAPO and 4-nitroaniline (4-NA) and with different capillary movement speed together with LED oven input current.

#	composition and LEDoven settings	profilometry images
1	1 % BAPO 2.5 mg/g 4-NA 5 mA 3 mm/s	
2	1 % BAPO 2.5 mg/g 4-NA 5 mA 5 mm/s	
3	1 % BAPO 2.5 mg/g 4-NA 10 mA 5 mm/s	
4	1 % BAPO 2.5 mg/g 4-NA 10 mA 3 mm/s	
5	0.5 % BAPO 0.9 mg/g 4-NA 7 mA 3 mm/s	
6	0.5 % BAPO 0.9 mg/g 4-NA 10 mA 3 mm/s	

By varying the amount of BAPO as initiator and 4-NA as the compound responsible for light attenuation and also by changing capillary movement speed and LED input it was possible to influence the thickness of the porous polymer at the capillary wall. Concentration of 4-nitroaniline was the critical parameter followed by speed of the speed of the capillary through the LED oven responsible for the dosage provided. The amount of initiator allowed to control the rate of polymerization, it was found to be useful to reduce BAPO concentration to 0.5 % in order to decrease sensitivity of polymerization mixture when lower concentration of 4-NA was used.

However, the previously observed issue with attachment of larger polymer particles leading to inhomogeneity of thickness distribution was still present, although its occurrence was much reduced compared to results obtained with inhibition by hydroquinone. That could be explained by sedimentation of larger polymer chains or particles in the direction of gravity which corresponds to location of a thicker layer at one side of the capillary wall (as shown on many profilometry images in Table 7, however, during manipulation with capillary cuts it was not possible to keep direction of the samples the same). For such reasons column-to-column reproducibility was considerably poor and probably length of the prepared column and correspondingly total time of the irradiation/polymerization process would be another factor having an impact on the results. Light attenuation approach was not pursued any further.

4.3.1.5 ITP-assisted gradient elution

Although the fabricated columns show inhomogeneity in thickness of the polymer over length of the capillary, column #6 was examined for use in CE for in-line preconcentration of chromate. SEM images of the column are shown on Figure 43.

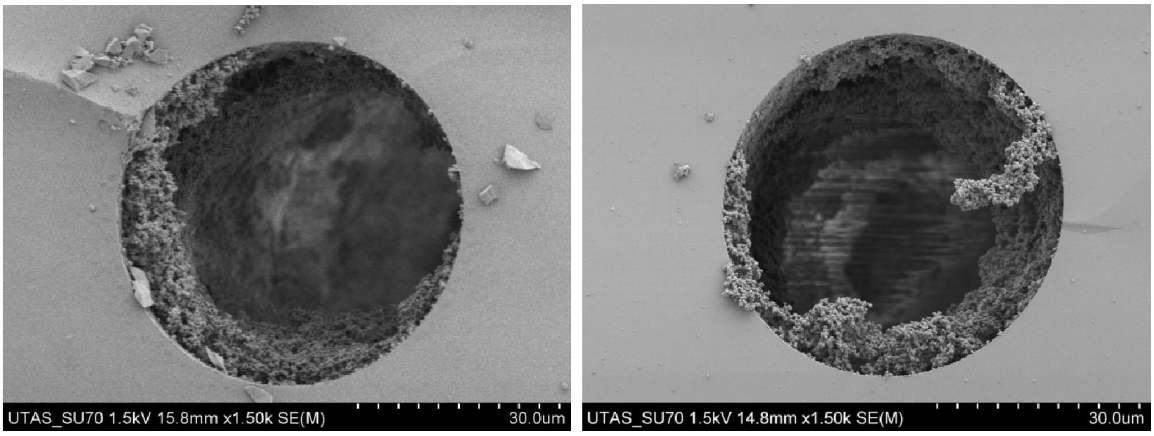


Figure 43. SEM images of cuts obtained from column #6 from Table 7.

A preconcentration method introduced by Breadmore *et al.* [124] was applied for analysis of chromate as a selected model compound. The approach is based on isotachophoresis-assisted gradient elution when the analyte has to fulfil two conditions: (i) its effective mobility has to be lower than mobility of the leading anion and at the same time faster than the terminating ion and (ii) ion-exchange selectivity coefficient of the analyte has to be higher than of the leading anion and lower than of the terminating anion.

The column pretreated with leading ion could be filled with large volume of sample and then sample is replaced with the weakly interacting leading electrolyte without elution of analyte strongly bound to the stationary phase. Finally, terminating electrolyte is placed at the capillary inlet and after application of voltage preconcentration occurs at the boundary with the terminating electrolyte.

4.3.1.5.1 Experimental conditions

γ-Methacryloxypropyltrimethoxysilane (γ-MAPS), glycidyl methacrylate (GMA), ethyleneglycol dimethacrylate (EDMA), phenylbis(2,4,6-trimethylbenzoyl)phosphine oxide, 1-dodecanol, cyclohexanol, tris(hydroxymethyl)aminomethane (Tris) were obtained from Sigma-Aldrich (St. Loius, MO, USA). Hydrochloric acid, perchloric acid, sodium hydroxide, ethanol, and methanol were from Ajax Chemicals (Seven Hills, Australia). In order to introduce quaternary ammonia functional group, the columns were photografted with [2-(methacryloyloxy)ethyl]trimethylammonium chloride (META). Capillary was filled with 15 % (m/m) META in 1-butanol similarly to conditions reported elsewhere [125] and passed through the LED oven at 3 mm/s with 5 mA LED input current. Capillary was washed with methanol. The extent of surface modification of META was not evaluated.

The analyses were performed by an HP^{3D} CE equipped with an UV-Vis diode-array detector. Teflon-coated fused-silica capillaries (Polymicro Technologies Inc., Phoenix, AZ, USA, 50 μm ID, 35 cm total length, effective length 26.5 cm) were used. Temperature of the capillary cassette was set to 25 ºC and voltage was -20 kV. BGEs was prepared by diluting perchloric acid to the desired concentration and pH was adjusted to value 8.0 using Tris.

4.3.1.5.2 In-line preconcentration of chromate

Strong anion exchange column was obtained by modification of the polymer surface by positively charged monomer. Chromate was selected as a model analyte; chloride as the leading ion and perchlorate as the terminating ion were found as suitable electrolyte components that fulfil the mobility and ion-exchange selectivity conditions according to published results [126] shown in Table 8. Steps of the method are described on schemes on Figure 44.

Table 8. Electrophoretic mobilities (μ_e) and ion-exchange selectivity coefficients using fluoride as a reference ($K_{E,F}$) for selected anions [126].

anion	μ_e [$\times 10^{-9}$ m^2/Vs]	$K_{E,F}$
fluoride	-58.12	1.0
chloride	-76.27	18.4
chromate	-72.23	857
perchlorate	-63.66	2350

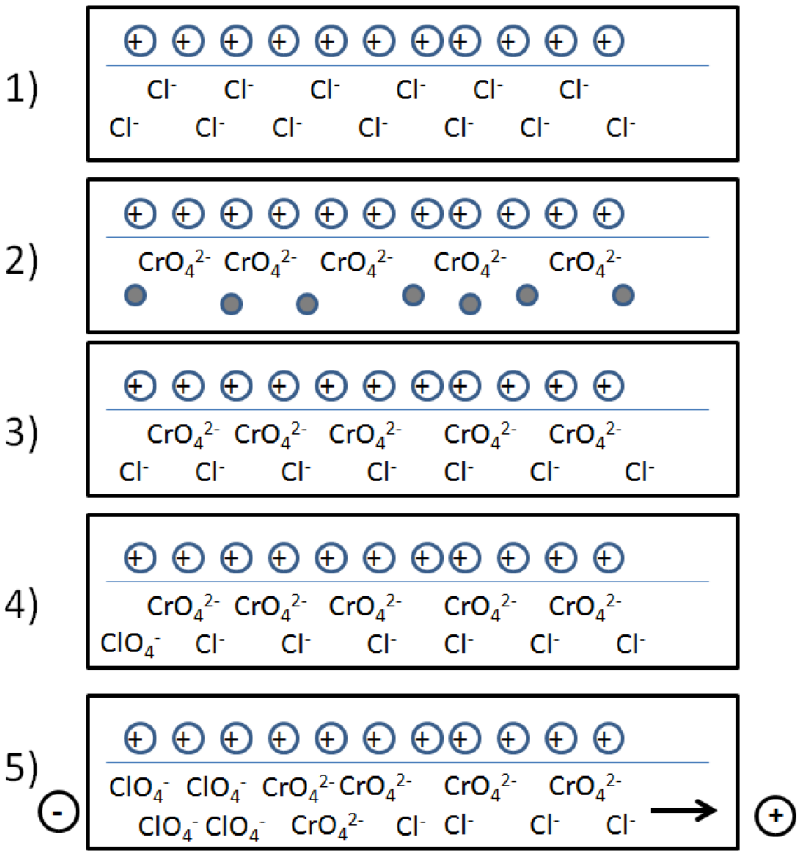


Figure 44. A scheme of ITP-assisted gradient elution for preconcentration of chromate.
(1) Capillary is filled with the weakly interacting electrolyte (chloride).
(2) Sample is introduced into the capillary, chromate is bound to the anion-exchanger.
(3) Sample solution is replaced by the weakly interacting electrolyte.
(4) The strongly interacting electrolyte is introduced at the inlet of the capillary.
(5) Voltage is applied, anions migrate towards anode and chromate is focused on a boundary with by the strongly interacting terminating electrolyte.

Thicker coating of column #6 probably led to blockage at some parts as it was not possible to wash the capillary within 20 minutes using internal pump with pressure of 1 bar. With use of external pressure of 4 bar, the volume of the column was replaced in about 4 minutes (based on a absorbance signal drop when washing with different BGEs). Sample injection had to be done using external pressure as well.

Based on mobilities and stability constants previously published [126], electrolytes were set as follows: capillary was filled with 10 mM NaCl and inlet and outlet vial contained 10 mM $HClO_4$ Tris pH 8.0. Between runs, capillary was flushed with 500 mM NaCl to regenerate the surface followed by 10 mM NaCl. Figure 45 shows an electropherogram of chromate sample analysis.

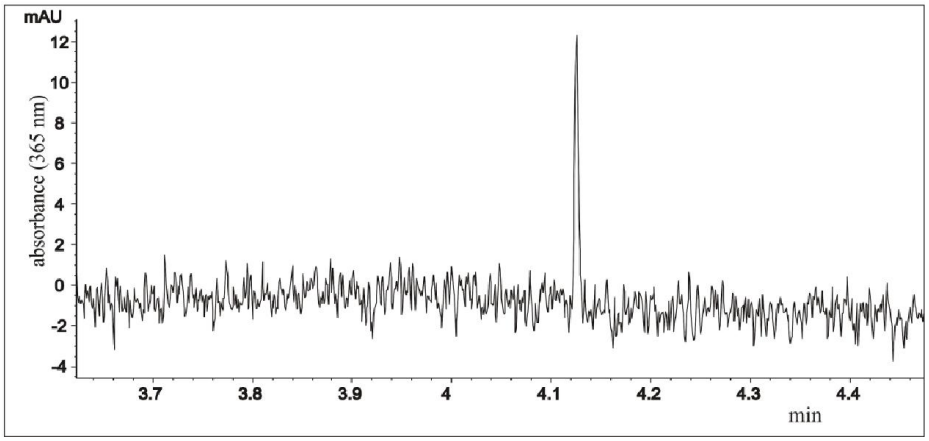


Figure 45. In-line preconcentration of chromate using column #6. Sample of 1 μM potassium chromate in water was injected at 4 bar for 180 sec.

Even though the column #6 showed obvious inhomogeneity in polymer thickness distribution, a sharp and symmetrical peak of chromate was observed. Higher baseline noise could be explained by thicker layer of the polymer coating present at the capillary window disturbing the absorbance measurement. When compared to analysis of chromate in an uncoated fused silica capillary using a standard injection of 50 mbar for 5 seconds, peak of a similar height was obtained with 300 μM potassium chromate.

4.3.2 Elimination of polymerization kinetics by immiscible liquid

Results shown in the section have been published in the following article:

- R. Knob, M.C. Breadmore, R.M. Guijt, J. Petr, M. Macka, *Porous layer open tubular monolith capillary column: switching-off the reaction kinetics as the governing factor in their preparation by using an immiscible liquid controlled polymerization*. RSC Advances, 3, 2013, 24927-24930.

In order to improve the inhomogeneity of the polymer thickness different approaches were investigated that rely on polymerization of whole length of the capillary at the same time. Adsorption of BAPO was on γ-MAPS modified capillary surface was evaluated, however, according to observed results the initiator was partly washed out when capillary was filled with the monomer mixture in the porogenic solvents (1-dodecanol, cyclohexanol). The beginning part of the capillary was left without any polymer attached whereas the end of capillary was filled with the polymer.

A modified experiment was performed based on replacement of the polymerization mixture from the centre of the capillary by a phase without monomers forming the polymer.

Several solvents were evaluated, e.g. cyclohexanol, 1-dodecanol, ethylacetate, ethyleneglycol, heptane or hexane. However, such solvents are more or less miscible with polymerization mixture constituents. For such reasons attention was brought to FC-770, a fully fluorinated solvent used e.g. as a coolant liquid for Beckman P/ACE MDQ instruments. Largely different physico-chemical properties of FC-770 from the constituents of the polymerization mixture ensure immiscibility.

4.3.2.1 Use of immiscible liquid

The process of replacement of the two liquids was monitored with use of optical microscope and images are shown on Figure 46. Figure 46A shows a boundary between the liquids in γ-MAPS modified capillary, the profile suggest superior wettability of the polymerization mixture (on the left side) over FC-770. Therefore after completely removing polymerization mixture in the capillary with FC-770 with flow rate of 100 µl/h

for 3 minutes, only a thin layer of polymerization mixture remains on the capillary wall as shown on Figure 46B.

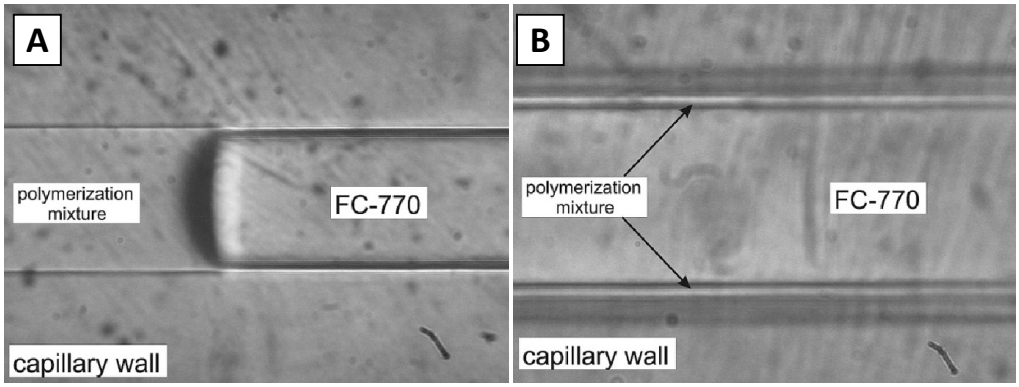


Figure 46. Optical microscope images of γ-MAPS modified capillary filled with polymerization mixture and FC-770. (A) shows a boundary between two liquids, (B) shows a thin layer of polymerization mixture left after replacement with FC-770.

After polymerization using a Shark series high-flux UV LED array with exposure for 1 minute, a homogeneous thin layer of porous polymer was obtained as shown on SEM images in Figure 47. Although only 0.25 μm thick polymer was produced, the thickness of the layer was maintained in several cuts which was a large improvement over the issues observed in previous approaches. However, such low thickness did not allow visualization by optical profiler.

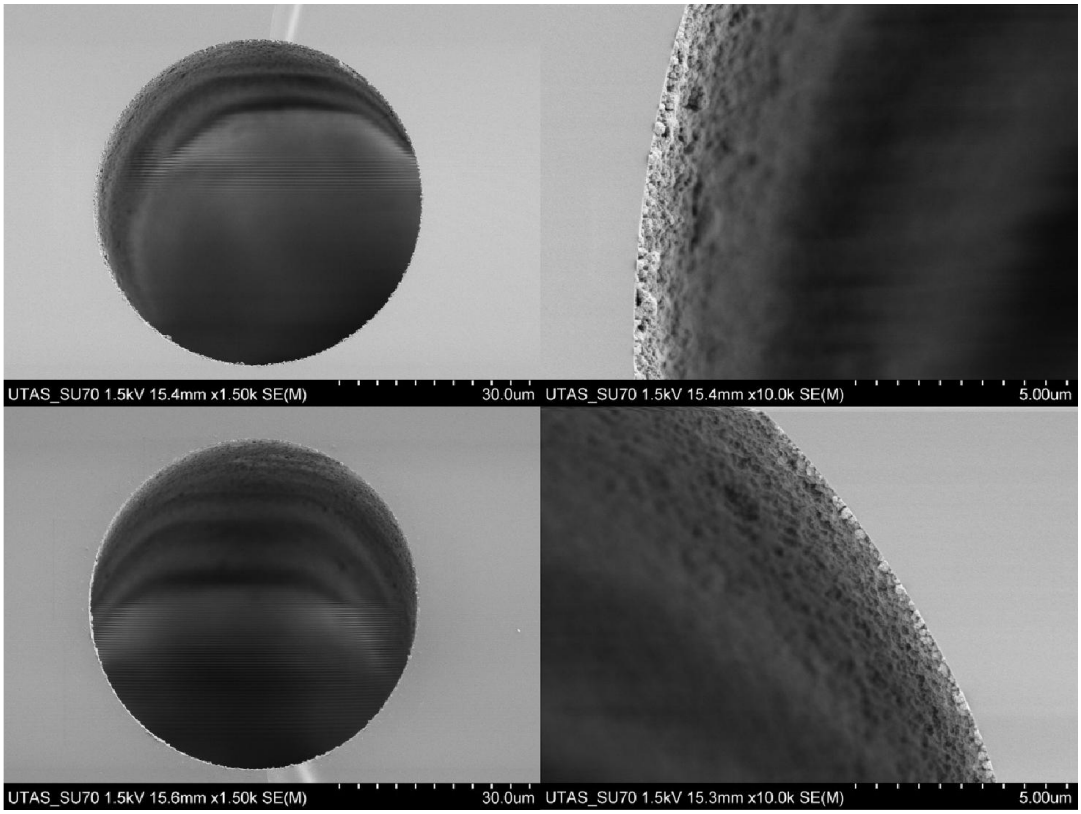


Figure 47. SEM images of two cuts of two different PLOT modified capillaries and corresponding details on right prepared using the approach based on replacement of the polymerization mixture by the immiscible liquid.

4.3.2.2 Capillary electrochromatography of inorganic anions

The applicability of prepared anion-exchange column was demonstrated on capillary electrochromatography of selected inorganic anions (chloride, bromide, nitrate) as model analytes.

4.3.2.2.1 Experimental conditions

Standards of bromide, iodide, and nitrate anions were prepared from their sodium salts of analytical grade (BDH Chemicals, Kilsyth, Australia). AS18 latex (quaternary ammonium functionalized) nanoparticles were supplied as an 11% (m/v) suspension by Dionex Corporation. All samples were prepared in water purified on a Millipore Milli-Q water purification system (Bedford, MA, USA).

Teflon-coated fused-silica capillary was modified with γ-MAPS and flushed with the polymerization mixture for 2 minutes at flow rate of 100 µl/h. The capillary was then flushed with FC-770 at flow rate of 100 µl/h for 3 minutes. After disconnecting the capillary from the syringe pump, it was placed under the UV Shark series high-flux LED array as 360 nm light source (OTLH-0480-UV, Opto Technology, Wheeling, IL, USA) with an output intensity of 0.8 mW/m^2.

The capillary was flushed with methanol for 30 min at a flow rate 100 µl/h followed by ring-opening the epoxy group by treatment with 0.1 M $NaHSO_3$ at 80˚C for 24 hours [127]. Finally, the capillary was flushed with water for 15 min. After coupling the PLOT capillary to the uncoated capillary, a 1% (m/v) aqueous suspension of Dionex AS18 anion-exchange latex particles was flushed through the capillary at 4 bars for 5 minutes. Next, the capillary was flushed with water and then with the BGE for 10 min each [128].

The capillary electrophoresis instrument used was an Agilent ^{3D}CE (Waldbronn, Germany) equipped with an external pressure device. Separations were carried out using 50 µm ID Teflon-coated fused-silica capillary (Polymicro Technologies Inc., Phoenix, AZ, USA). A 10 cm piece of porous polymer modified capillary was coupled via a 0.5 cm polytetrafluoroethylene sleeve to 25 cm of polyamide coated fused-silica capillary. Separation voltage was 20 kV. Detection was performed 8.5 cm from the anode through the PTFE-coated capillary. The capillary cassette was thermostated to 25˚C. The BGE was prepared by diluting perchloric acid to the desired concentration and adjusting the pH to 8.0 using tris(hydroxymethyl)aminomethane (Tris).

4.3.2.2.2 Separation of inorganic anions

Figure 48 shows electropherograms of the model mixture using perchlorate Tris-based BGE of pH 8.0. In case of 10 mM BGE, the strongest interactions with the latex nanoparticles modified PLOT column were obtained which allowed iodide to be retained considerably more than the other two ions resulting in a change in selectivity.

Increasing the concentration of the BGE increases the elution strength of the electrolyte which reduces the extent of interaction with the PLOT thus the effective mobility is increased. In 20 mM BGE, iodide co-migrates with nitrate (Figure 48, middle),

with higher BGE concentration further reducing interaction with the PLOT and a baseline separation is achieved (Figure 48, right). In comparison, the same separation in an uncoated capillary without modification by the quaternary ammonia latex nanoparticles is shown in Figure 49. Co-migration of bromide and iodide was observed due to the absence of any ion-exchange interactions. The lower and higher concentration of the BGE did not bring any further changes in the separation order as was the same as in the PLOT column.

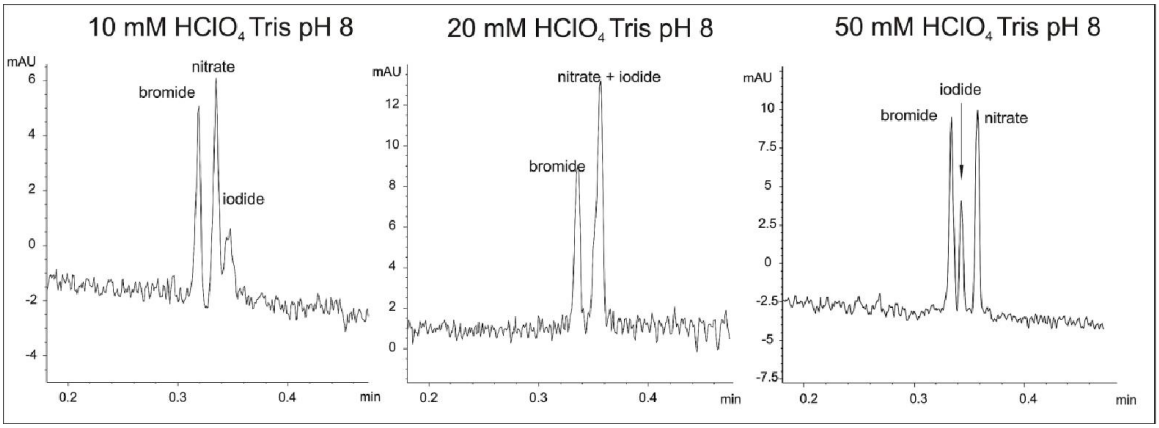


Figure 48. Separation of selected inorganic anions at 0.1 mM using modified anion exchange PLOT column and the BGEs with various elution strength. Voltage 20 kV, injection for 3 s at 50 mbar at short end of the column. Detection at 200 nm.

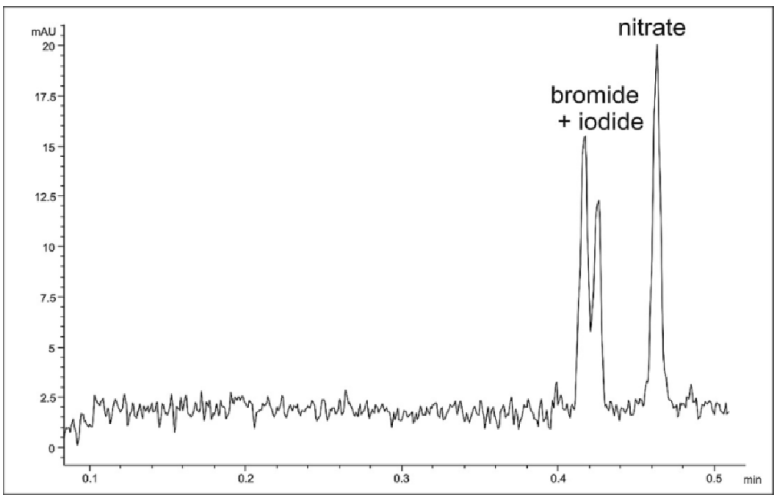


Figure 49. Separation of selected inorganic anions using unmodified silica capillary and the BGE of 10 mM $HClO_4$ Tris pH 8.0. Conditions are the same as on Figure 48.

Repeatabilities of bromide, iodide, and nitrate migration time are summarized in Table 9.

Table 9. Repeatabilities of migration time of bromide, iodide, and nitrate (in % RSD) using modified PLOT column and 10 mM $HClO_4$ Tris pH 8.0.

	bromide	**iodide**	**nitrate**
intraday (n=5)	0.22	0.49	0.46
interday (n=3)	1.00	1.41	0.96

4.3.2.2.3 Comparison with other columns

Next, results obtained with latex nanoparticles modified PLOT column were compared with previously reported open tubular column modified with the same nanoparticles [124] regarding effective mobilities of the selected inorganic anions and corresponding capacity factor (*k*') calculated by:

$$k' = \frac{\mu_{eff,bare} - \mu_{eff,column}}{\mu_{eff,bare}} \quad (9)$$

where $\mu_{eff,bare}$ presents effective mobility in an uncoated capillary without any cation-exchange functional groups and $\mu_{eff,column}$ presents effective mobility obtained on latex nanoparticles modified column. Table 10 shows the comparison of three columns with different surface modifications and migration parameters. Using 10 mM $HClO_4$ Tris pH 8.0 as the BGE, increase of *k*' for the PLOT columns compared with the open tubular was 11.5 % for bromide, 20.1 % for iodide, and 8.9 % for nitrate.

Table 10. Effective mobilities and capacity factors of bromide, iodide, and nitrate in an uncoated capillary, open tubular [124], and PLOT modified columns using 10 mM $HClO_4$ Tris pH 8.0.

		bromide	**iodide**	**nitrate**
μ_{eff} [$\times 10^{-9}$ m^2/Vs]	uncoated	-81.2	-79.6	-75.2
	open tubular	-71.6	-64.7	-66.2
	PLOT	-70.5	-61.7	-65.4
k'	open tubular	0.118	0.187	0.120
	PLOT	0.132	0.225	0.130

4.3.3 Conclusion

Various approaches for fabrication of PLOT columns were investigated using LED-based device for photopolymerization of porous polymers as stationary phase for capillary electrochromatography applications. Influence on polymerization kinetics by addition of hydroquinone as the radical scavenger and utilization of 4-nitroaniline as the light-attenuating agent were tested, however limitations with unsatisfactory homogeneity of the coating thickness were observed when longer columns were prepared.

Optical profilometry was utilized for PLOT column inspection in 50 μm ID capillaries for assessment of the thickness and homogeneity of the produced coating. The technique was found as a useful alternative to SEM for its less demanding requirements on sample preparation and immediate availability to use. However, limitations in lateral resolution (when lens with 40x magnification was used) of about 1 μm were noted that did not allow full assessment of such thin coatings.

In-line preconcentration of chromate as a model analyte was performed using ITP-assisted gradient elution. The results show that such preconcentration method can be successfully used even though the column showed significant inhomogeneity in the thickness distribution of the stationary phase.

A new approach for preparation of PLOT columns in Teflon-coated silica capillaries was developed. The polymerization mixture is expelled from the centre of the capillary by the immiscible liquid, FC-770, leaving only a thin layer of polymerization mixture on the capillary wall. Such procedure eliminates issues with incomplete polymerization that were found with previous approaches, however, only a thin 0.25 μm layer was formed.

The applicability of the prepared columns modified with anion-exchange latex nanoparticles was demonstrated on the separation of three selected inorganic anions: bromide, iodide, and nitrate, that was achieved within 22 seconds. By changing the elution strength of the BGE it was possible to change the migration order of analytes. Improvement in capacity factors over previously reported open tubular column was found as 11.5 % for bromide, 20.1 % for iodide, and 8.9 % for nitrate.

5. CONCLUDING REMARKS

In conclusion, so far amazing variety of capillary coatings has been developed and reported to use, they are reflected in the theoretical part of the thesis. However, there is no such versatile procedure that can be generally used for every application as different aims, surface properties or effects are desired.

The experimental part of the thesis was focused on separation of compounds with pharmaceutical and physiological relevance using various capillary coatings. They prevented analyte adsorption on the capillary wall and allowed fast analysis and good repeatability of migration times, which is one of the often mentioned limitation of capillary electrophoresis for its wider employment. Specific aspects of each of three parts of the experimental part of the thesis are summarized in the corresponding section.

Manipulation of migration order was studied by using different coatings that allowed to tune the EOF direction and magnitude, also in chiral separation the use of different chiral selectors allowed to change the migration order.

A new approach for fabrication of thin layer PLOT column based on use of immiscible liquid was developed. Optical profilometry was used for the first time for assessment of the layer thickness offering much faster and easier sample preparation than scanning electron microscopy. Together with increased capacity in comparison to open tubular columns, such columns offer fast rinsing times and has no requirements on pumps. The procedure allows preparing the homogeneous coating as the stationary phase for capillary electrochromatography or possibly for other applications that would benefit from high permeability. Compatibility with other polymerization mixtures and initiators, a possibility of production thicker coatings or deposition of multiple layers will be certainly studied in the near future.

LIST OF ABBREVIATIONS

4-NA	4-nitroaniline
AIBN	azobisisobutyronitrile
BAPO	bis(2,4,6-trimethylbenzoyl)-phenylphosphineoxide
BGE	background electrolyte
CCD	contactless conductivity detection
CE	capillary electrophoresis
CEC	capillary electrochromatography
CGE	capillary gel electrophoresis
CZE	capillary zone electrophoresis
dcSAH	decarboxylated *S*-adenosylhomocysteine
dcSAM	*S*-adenosyl-(5)-3-methylthiopropylamine
DMPAP	2,2-Dimethoxy-2-phenylacetophenone
EACA	ε-aminocaproic acid
EDMA	ethylene dimethacrylate
EKC	electrokinetic chromatography
EOF	electroosmotic flow
ESI	electrospray ionization
GC	gas chromatography
GMA	glycidyl methacrylate
ID	internal diameter
IEF	isoelectric focusing
ITP	isotachophoresis
LC	liquid chromatography
LED	light emitting diode
LIF	laser-induced fluorescence detection
LOD	limit of detection
L-OH-PRO	*trans*-4-hydroxy-L-proline
LOQ	limit of quantitation
M7C4I	1-(4-iodobutyl)-4-aza-1-azoniabicyclo[2,2,2] octane iodide

MEMS	micro-electro-mechanical systems
META	[2-(methacryloyloxy)ethyl]trimethylammonium chloride
MIP	molecularly imprinted polymer
MOF	metal-organic frameworks
MS	mass spectrometry
MTA	5'-methylthioadenosine
NMR	nuclear magnetic resonance
OD	outer diameter
PLOT	porous layer open tubular
PS-DVB	poly(styrene-*co*-divinylbenzene)
PVPy-Me	*N*-methylpolyvinylpyridinium
SAH	*S*-adenosyl-L-homocysteine
SAM	*S*-Adenosyl-L-methionine
SEM	scanning electron microscopy
SI-ATRP	surface initiated atom transfer radical polymerization
TEMED	tetramethylethylenediamine
TEOS	tetraethoxysilane
Tris	tris(hydroxymethyl)aminomethane
UV	ultraviolet
γ-MAPS	γ-methacryloxypropyltrimethoxysilane

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CURRICULUM VITAE

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Education:

2005 - 2008	Bachelor study of chemistry, Faculty of Science, Palacký University in Olomouc, defense of bachelor thesis: *Separation of organic acids by capillary electrophoresis with contactless conductivity detection*
2008 - 2010	Master study of analytical chemistry, Faculty of Science, Palacký University in Olomouc, defense of diploma thesis: *Polypyrrole coated capillaries for capillary electrophoresis*
2012	Post-graduate doctoral degree (RNDr.), Faculty of Science, Palacký University in Olomouc, defense of rigorous thesis: *Analýza vybraných bromovaných fenolů pomocí on-line spojení kapilární izotachoforézy s kapilární zónovou elektroforézou* (Analysis of selected brominated phenols by on-line coupled capillary isotachophoresis and capillary zone electrophoresis)
2010 - till now	Ph.D. study of analytical chemistry, Faculty of Science, Palacký University in Olomouc, topic: Study on capillary coatings for capillary electrophoresis

Research / Work Experience:

2008	Research stay, Chair of Environmental Chemistry and Ecotoxicology (supervisor: prof. Hartmut Frank), University of Bayreuth, Germany, February – April.
2009 - 2011	Vocational work, TEVA Czech Industries, Opava-Komárov, Solid-state R&D (supervisors: Dr. Aleš Gavenda and Dr. Aleš Husek), five visits, 4 months in total.

2009	Research stay, Department of Analytical Chemistry (supervisor: Ass. prof. Jozef Marák), Faculty of Natural Science, Comenius University in Bratislava, Slovakia, May – June.
2010	Research stay, Dipartimento di Chimica (supervisor: prof. Pier Giorgio Righetti), Politecnico di Milano, Italy, February – May.
2011 - 2012	Honorary Research Associate at the Australian Centre for Research on Separation Science and School of Chemistry, University of Tasmania, Hobart, Australia, September - May, supervisor: prof. Mirek Macka.
2012 - 2013	Research work, Department of Forensic Medicine and Medical Law, Faculty of Medicine, Palacký University in Olomouc (supervisor: Ass. prof. Peter Ondra), October - June.
2013	Research stay, KIST Europe (supervisor: prof. Andreas Manz), Saarbrücken, Germany, April – May.
2013	Research stay, Australian Centre for Research on Separation Science, University of Tasmania, Hobart, Australia (supervisor: prof. Michael Breadmore), November - December.
2014	Research stay, KIST Europe (supervisor: prof. Andreas Manz), Saarbrücken, Germany, March.

Employment / Projects

2010 - 2014	Student grant competition, IGA Palacký University in Olomouc, member of the team
2011 - 2014	Kontakt LH11018, *Cyclofructans and their derivatives as new chiral selectors for liquid chromatography and capillary electrophoresis*, member of the team
2012	University Development Fund *(FRVŠ)*, *New laboratory exercises utilizing non-aqueous solvents*, principal investigator
2012 – 2014	IGA Ministry of Health of the Czech Republic NT1393-3/2012, *Diagnostics of intoxication by designer drugs*, member of the team
2012 - 2014	Operational Program Education for Competitiveness CZ.1.07/2.3.00/20.0018, *Development of human resources for excellence in research in nanotechnology in analytical chemistry*, member of the team

2012 - 2014	Operational Program Education for Competitiveness CZ.1.07/2.4.00/17.0065, *Partnership and networks for an excellence in chemistry*, member of the team
2012 - 2014	Operational Program Education for Competitiveness CZ.1.07/2.4.00/31.0006, *Development of research and education in chemistry and medicine of burn injuries,* member of the team
2013	University Development Fund *(FRVŠ)*, N*ew laboratory exercises of food analysis by capillary electrophoresis*, principal investigator

Teaching:

2011	Laboratory course of analytical chemistry for 2nd year students of bachelor degree, 1 semester
2012	Laboratory course of analytical and environmental chemistry for 2nd year students of bachelor degree, 1 semester

Publication activity:

[1] V. Maier, J. Petr, R. Knob, J. Horáková, J. Ševčík: *Electrokinetic partial filling technique as a powerful tool for enantiomeric separation of D,L-lactic acid by capillary electrophoresis with contactless conductivity detection*. Electrophoresis **28** (2007) 1815.

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Book chapters:

R. Knob, M. Macka, P.R. Haddad: *Capillary Electrochromatography* in *Encyclopedia of Analytical Science*, Third edition, Elsevier 2013.

Conference oral presentations during Ph.D. study::

[1] R. Knob, M.C. Breadmore, R. Guijt, J. Petr, M. Macka: *Control of the polymerization towards porous layer open tubular capillary columns*. CECE 2012, 1. - 2. November 2012, Brno, Czech Republic.

[2] R. Knob: *Analýza nečistot farmaceutických substancí*. Medicína a chemie popáleninových stavů, 27. Februrary 2014, Ostrava, Czech Republic.

Conference poster presentations during Ph.D. study:

[1] R. Knob, V. Maier, J. Petr, V. Ranc, J. Ševčík: *First time capillary electrophoretic separation of perfluorooctanoic acid and perfluorooctanesulfonic acid in non-aqueous media*, Nordic Separation Science Society 6th Conference, 24. – 27. 8. 2011, Riga, Latvia.

[2] R. Knob, V. Maier, J. Petr, V. Ranc, J. Ševčík: *Possibilities of on-line preconcentration of perfluorooctanoic acid and perfluorooctanesulfonic acid by non-aqueous capillary electrophoresis*, 18th International Symposium on Electro- and Liquid Phase-separation Techniques (ITP 2011), Tbilisi, Georgia, 28. - 31. August 2011.

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Popularization activities of Department of Analytical Chemistry at Faculty of Science, Palacký University in Olomouc

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PALACKÝ UNIVERSITY IN OLOMOUC

Faculty of Science

Department of Analytical Chemistry

SUMMARY

OF THE DOCTORAL THESIS

Coatings for capillary electrophoresis

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Field of study: Analytical chemistry

Supervisor: prof. RNDr. Juraj Ševčík, Ph.D.

OLOMOUC 2014

SUMMARY

Surface modifications of fused silica capillaries for use in capillary electrophoresis have been studied. Such coatings are essential in situations when it is necessary to prevent analyte adsorption that may lead to peak tailing and deterioration of the separation. They are also found useful for changing the direction or magnitude of the electroosmotic flow, or when interactions with the surface are desired for the purpose of chromatographic retention that influences the separation.

A number of various approaches have been introduced including dynamic or covalent modifications of the capillary surface determining its suitability for certain application. In this work, three demonstrations of capillary coatings for separation of small molecules by means of capillary electrophoresis are described.

For analysis of diastereoisomers of decarboxylated impurities related to *S*-adenosylmethionine a dynamic coating based on the background electrolyte containing barium borate was utilized. Such modification allowed to reduce the magnitude of the electroosmotic flow at alkaline pH and separation of the diastereoisomers with resolution of 1.35 was achieved in 10 minutes. The developed method allowed determination of the impurities in highly concentrated sample of *S*-adenosylmethionine without interference of the main component.

Separation of tartaric acid enantiomers with use of contactless conductivity detection was achieved in polyacrylamide coated capillary as it was necessary to suppress the electroosmotic flow directed against migration of tartaric acid. Complex of copper(II) and *trans*-4-hydroxy-L-proline was used as a chiral selector, resolution of 1.9 and limit of detection about 20 μM was achieved, however, baseline separation could be maintained only till enantiomeric ratio of 1D : 7L. Use of various counter-ions in the background electrolyte was studied and reversal of migration order of the enantiomers was observed. The method was applied on analysis of tartaric acid in wine, grape juice, and pharmaceutical substances containing tartaric acid as a counter-ion to the active substance. Only L-tartaric acid was detected.

The last part of the thesis is devoted to the fabrication of porous layer open tubular columns. An approach of delivering the capillary through a device equipped with LEDs for photoinitiated polymerization of porous polymers was pursued, however, even after modification of the polymerization mixture with a radical scavenger and a light-attenuating agent difficulties related with inhomogeneity in thickness distribution were still encountered. Optical profilometry was used together with scanning electron microscopy for assessment of the layer thickness. Improved results regarding the homogeneity of the coating were achieved with an approach based on replacement of the polymerization mixture by an immiscible liquid from the centre of the capillary followed by photoinitiated polymerization. Such procedure leads to formation of about 0.25 μm thick porous layer completely shielding the silica surface. Such modified capillaries were evaluated by anion-exchange capillary electrochromatography of selected inorganic anions enabling changing the migration order of iodide and nitrate by adjustment of the elution strength of the background electrolyte. Improvement in capacity factors over previously reported open tubular column was achieved, ranging from 8.9 % to 20.1 %.

SOUHRN

V dizertační práci byly studovány modifikace povrchu křemenných kapilár pro použití v kapilární elektroforéze. Pokrytí stěny kapiláry je potřebné pro zabránění adsorpce analytů na povrch kapiláry, které může vést k chvostování píků a tím zhoršení separace. Dále jsou modifikace povrchu využívány pro změnu směru a rychlosti elektroosmotického toku, nebo pokud je žádána chromatografická retence pro ovlivnění separace.

V minulosti bylo vyvinuto množství způsobů pokrytí stěny křemenných kapilár, často jsou založeny buď na dynamické, nebo kovalentní modifikaci povrchu; vhodnost využití těchto způsobů závisí především na povaze použití. V této práci jsou představeny tři případy použití pokrytí povrchu s využitím pro separaci malých molekul kapilární elektroforézou.

Dynamické pokrytí využívající barnaté kationty a kyselinu boritou jako složky základního elektrolytu bylo využito pro separaci diastereoizomerů dekarboxylovaných nečistot *S*-adenosylmethioninu. Tato modifikace povrchu dovolila snížení rychlosti elektroosmotického toku v zásaditém základním elektrolytu. Separace diastereoizomerů s rozlišením 1,35 byla dosažena během 10 minut. Vyvinutá metoda byla použita pro analýzu této nečistoty ve velmi koncentrovaném vzorku *S*-adenosylmethioninu, jehož nadbytek nerušil při stanovení.

Kovalentní pokrytí polyakrylamidem pro eliminaci elektroosmotického toku bylo využito pro separaci enantiomerů vinné kyseliny s využitím bezkontaktní vodivostní detekce. Komplex měďnatých kationtů a *trans*-4-hydroxy-L-prolinu byl použit jako chirální selektor. Bylo dosaženo separace enantiomerů s rozlišením 1,9 s detekčním limitem přibližně 20 μM; nicméně separace na základní linii bylo možné dosáhnout pouze u vzorků, kde poměr enantiomerů byl nejvýše 1D : 7L. Použití různých protiiontů základního elektrolytu umožnilo změnit migrační pořadí enantiomerů. Metoda byla použita pro stanovení vinné kyseliny ve víně, hroznové šťávě a farmaceutik, která obsahují vinnou kyselinu jako protiiont k aktivní substanci. Ve vzorcích byl detekován pouze L-enantiomer.

Poslední část práce byla věnována přípravě *"porous layer open tubular"* kolon. Byly zkoumány možnosti protahování kapiláry skrz zařízení vybavené

polovodičovými diodami (LED), které sloužilo pro světelně spouštěnou polymeraci pro modifikaci povrchu porózním polymerem. Nicméně i při využití inhibitoru radikálové polymerace nebo látky zeslabující průnik světla do roztoku nebylo dosaženo uspokojivých výsledků co se týče homogenity distribuce tloušťky polymeru na stěně kapiláry. Kromě skenovacího elektronového mikroskopu byla využita také optická profilometrie pro zhodnocení tloušťky polymeru. Lepších výsledků bylo dosaženo promytím kapiláry kapalinou nemísitelnou s polymerační směsí zanechávající pouze tenkou vrstvu polymerační směsi na stěně kapiláry. Po její polymeraci bylo připraveno pokrytí o tloušťce přibližně 0,25 μm rovnoměrně pokrývající stěnu kapiláry. Modifikované kolony byly využity pro separaci modelové směsi anorganických iontů pomocí kapilární elektrochromatografie s anexovou stacionární fází. Změna eluční síly základního elektrolytu umožnila změnu migračního pořadí jodidů a dusičnanů. Bylo dosaženo zlepšení kapacitních faktorů oproti dříve připraveným *"open tubular"* kolonám o 8,9 až 20,1 %.

TABLE OF CONTENTS

INTRODUCTION

Coatings for capillary electrophoresis (CE) play an important role in its applications for analysis of various compounds when the surface properties and repeatability of its conditions of widely used bare fused silica capillaries are not suitable. Modifications of the capillary surface could be performed in a number of approaches utilizing large spectra of compounds dealing differently with specific problems as analyte adsorption or changing electrokinetic properties of silica surface; or on the other hand mediating desired interactions with the surface as a stationary phase that largely influences selectivity of the separation.

Various additives as small molecules, polymers or particles have been introduced to alter separation parameters allowing e.g. much faster analysis by control over generation of electroosmotic flow (EOF) direction and magnitude as well. A number of approaches have been developed for fabrication of porous polymer layers that enhance the surface area with different surface chemistries area allowing increased retention of molecules or more effective immobilization of various objects.

There is no such procedure that can be utilized in every situation, therefore for specific applications various approaches have been developed that reflect desired properties of the coating.

The necessity of characterization of the developed capillary coatings have brought attention to methods of assessment of coating properties and durability showing different applicability of the developed approaches for certain applications.

CAPILLARY COATINGS

In CE wall modifications leaving the profile of the capillary open are the most frequently used. Open tubular format allows easy washing and injecting into the capillary which is beneficial as most of CE instrument lack high pressure pump. A large number of chemical substances have been introduced, both dynamically or covalently attached onto the capillary wall.

A number of compounds can be utilized as dynamic coatings agents that can be used for modification just by rinsing the capillary with their solution or by their presence in the background electrolyte (BGE) during analysis. Small molecules like diamines [1], cationic surfactants [2] and ionic liquids [3] have been used. On the other hand, neutral or basic polymers could be adsorbed on the silica surface via several types of interactions as hydrogen bonding, Van der Waals interactions or coulombic forces [4].

Formation of permanent covalent bond between surface and the coating agent of capillary is another approach for surface modification of the capillary surface. Although more time and effort is required for preparation, improvement in lifetime and stability is usually obtained and such coatings allow changing the surface properties without presence of any additives in the BGE. Bifunctional silanes are mostly used to modify silica surface where the alkyloxy part is responsible for bonding with the silanol groups and the other functional group [5,6] determines modified surface properties, or it can be used as an anchoring support for polymers [7-9].

Thin coating layers (in range of nanometers) often result in low capacity and thus hamper chromatographic applicability in case when large surface area is needed. Porous layer open tubular (PLOT) columns have been introduced to overcome the above mentioned limited capacity. Although the solid to liquid ratio for PLOT columns is lower compared to packed or monolithic columns of the same inner diameter, their capacities are still much greater compared to open capillaries and sufficient for many applications. By using narrower capillary diameters, acceptable efficiencies can be achieved [10].

Many approaches for fabrication of PLOT columns have been reported. Some of them are based on formation of porous silica layer at the capillary wall by sol-gel method [11,12]. Methacrylate- and styrene-based porous polymers have become

popular during past two decades, several methods for fabrication of monolithic-like PLOT columns have been published using mostly thermal initiated free radical polymerization [13,14] followed by methods relying on photo-initiation [15,16]. An effect on capillary internal diameter was found to play an essential role having a large impact on morphology of the resulting polymer [17].

So far no general procedure has been reported for fabrication of PLOT columns that could be applied to any chemistry. Most of the methods rely on controlling the kinetics of the polymerization leading to incomplete polymerization that is hardly repeatable.

AIMS OF THE THESIS

The aim of the thesis was to explore possibilities of employment of various capillary coatings in applications of capillary electrophoresis to both reduce and support interactions with the capillary surface. Such modifications should be used in order to improve analysis performance regarding analysis time and resolution of the analytes.

The specific aims of the thesis could be divided into three parts:

i. Apply capillary coatings in order to prevent adsorption of basic compounds as derivatives of *S*-adenosylmethionine to prevent peak tailing.
ii. Apply covalent capillary coatings for control of the electroosmotic flow direction and magnitude in separation of tartaric acid enantiomers .
iii. Explore possibilities of formation of a stationary phase for capillary electrochromatography with increased surface area over classical open tubular format.

RESULTS AND DISCUSSION

- R. Sebastiano, R. Knob, A. Citterio, P.G. Righetti, *Analysis of trace degradation products (decarboxylated diastereoisomers) of S-adenosylmethionine by electrophoresis in capillaries with cationic coatings (N-methylpolyvinylpyridinium or divalent barium).* Electrophoresis 31, 2010, 3592-3596.

Analysis of decarboxylated products of *S*-adenosylmethionine

S-Adenosyl-L-methionine (SAM) belongs to group of biologically active compounds primarily responsible for transfer of a methyl group during metabolic processes. In fact, it is the only sulfonium cation present in mammals' body, SAM takes part in numerous methyltransferase reactions aimed on modification of various types of metabolites and macromolecules as proteins, nucleic acids, and polysaccharides. SAM exists in two diastereoisomeric forms, *S*,*S*- and *R*,*S*-adenosylmethionine, where the designations refer to the stereochemical configurations of the sulphur and the α-carbon. Only the *S*,*S*-form is biologically active; in mammals there is only a minor fraction of the *R*,*S*-SAM.

SAM can be decarboxylated by formation of *S*-adenosyl-(5)-3-methylthiopropylamine (dcSAM) and even this compound can exist in two diastereoisomeric forms. Structures of these two compounds are shown in Figure 1. These diverse forms of heterogeneity have called for methods for the rapid and accurate determination of SAM and of possible metabolites.

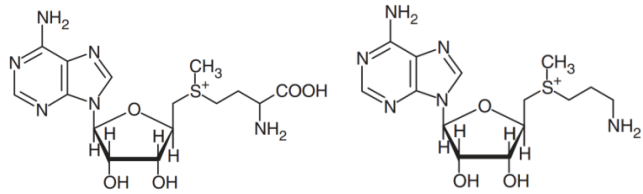

Figure 1. Structure of *S*-adenosyl-L-methionine (left) and *S*-adenosyl-(5)-3-methylthiopropylamine, dcSAM (right).

In the first experiments in an uncoated capillary, ammonium formate based BGE was selected for separation of dcSAM from the main sample component, SAM. The BGE was chosen reflecting further aim to transfer the method for CE-MS experiments in order to confirm peak identity with data obtained from MS. As separation of only diastereisomers of dcSAM was desired, no chiral selector was added into the BGE as separation of enantiomers producing up to 4 signals could be expected. Due to absence of standards it would be impossible to assign identity of related signals to corresponding diastereisomeric forms of dcSAM.

Analysis of the SAM sample at concentration of 5 mg/ml in 100 mM ammonium formate pH 4.0 is shown on Figure 2. Peak of dcSAM looked as a single symmetrical peak, therefore no hint of diastereisomers separation was observed. In the BGEs with pH higher than 6 the resolution between dcSAM and SAM peaks increased, however, due to strong interaction of these basic compounds with silanol groups of the silica capillary the separation became more deteriorated due to significant peak tailing.

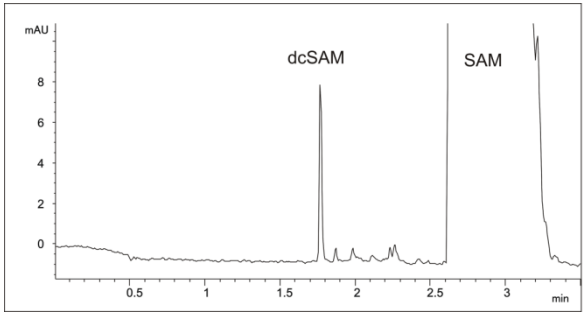

Figure 2. Analysis of SAM sample at concentration 5 mg/ml in 100 mM ammonium formate pH 4.0 using uncoated silica capillary.

Modification by *N*-methylpolyvinylpyridinium

In order to decrease effective mobilities and provide longer separation time for separation of dcSAM diastereoisomers, modification of the capillary wall by cationic polymer, *N*-methylpolyvinylpyridinium (PVPy-Me), was used [18,19].

The surface modification led to deposition of positively charged polymer on the capillary wall with the result of reversing the EOF direction towards anode. Using 50 mM ammonium formate pH 4 as the BGE, the electroosmotic mobility was determined as -32.5×10^{-9} m^2/Vs, measured at -20 kV with acrylamide as the EOF marker.

Analysis of SAM sample at concentration 5 mg/ml is shown on Figure 3. The prolongation the migration time allowed the diastereoisomers to partly separate as apparent from the doublet peak between 9 and 10 min. However, the large excess of SAM migrating in this case in front of dcSAM competed with the cationic polymer coating on the capillary surface (it was necessary to reapply polymer coating procedure to obtain repeatable results) and thus diminished separation of the diastereoisomers due to observed peak tailing.

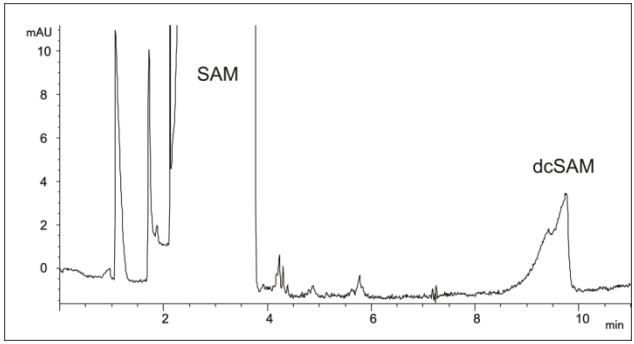

Figure 3. Analysis of SAM sample at concentration 5 mg/ml in 50 mM ammonium formate pH 4.0 using PVPy-Me modified capillary.

Barium borate modification

In another approach, dynamic modification with the BGE based on barium borate was examined. Barium salts were noted as effective additives to suppress EOF even at higher pH values [20].

The BGE based on 50 mM boric acid adjusted with barium hydroxide (20 mM) was tested; however, observed electroosmotic mobility of -12.1×10^{-9} m^2/Vs almost matched effective mobility of dcSAM (8.5×10^{-9} m^2). EOF directed oppositely to dcSAM migration would unreasonably extend the analysis time. Therefore, different ratios of barium and sodium borate buffers were mixed and tested. The expected effect of sodium cations was to counterbalance the influence of barium cations to reverse of migration, therefore by fine-tuning their ratio it would be possible to precisely manipulate with electroosmotic mobility.

Best results regarding resolution of dcSAM diastereisomers were achieved in the BGE composed of 50 mM boric acid, 10 mM sodium hydroxide, 10 mM sodium hydroxide (pH 9.4), Figure 4 shows electropherogram of the separation.

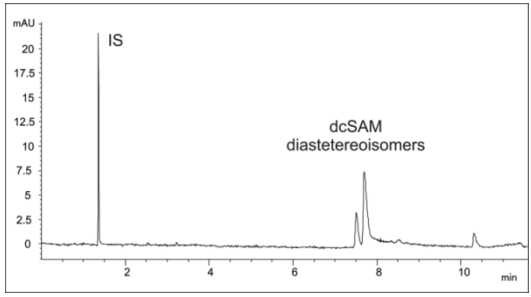

Figure 4. Analysis of SAM sample at concentration 40 mg/ml using barium borate based BGE. *N*-methylpyridinium iodine (0.3 mg/ml) was used as an internal standard (IS).

Enantioseparation of tartaric acid

- R. Knob, J. Petr, J. Ševčík, V. Maier, *Enantioseparation of tartaric acid by ligand-exchange capillary electrophoresis using contactless conductivity detection*. Journal of Separation Science 36, 2013, 3426-3431.

Tartaric acid is dicarboxylic acid possessing two hydroxy groups, it is a chiral compound, it has two chiral centers located at the carbon atom linked with hydroxy and carboxylic functional groups.

In nature, L-tartaric acid is the mostly occurring, it is distributed in plants and part of beverages as juices, wine, sake, etc. Oppositely, D tartaric acid is much rare to find, some of *cis*-epoxysuccinate hydrolase-containing bacteria are able to produce it [21]; it also found use in pharmaceutical industry as a chiral intermediate. D-tartaric scarcely occurs in nature, it can be considered as a marker of a bacterial contamination of beverages. The enantiomeric purity of tartaric acid used as a food additive is required for safety and quality control of the products. Similarly, enantiomers of tartaric acid declared as a counterion of several pharmaceutical substances has to be controlled due to their potential adverse physiological effects.

Covalent capillary modification by polyacrylamide

Modification of the capillary surface was considered due to anionic character of tartaric acid and expected EOF magnitude in an uncoated capillary in slightly acidic BGE that would be directed against the migration of the analyte.

The surface modification was carried on according to previously published procedure with several modifications [22]. Capillary was first pretreated with sodium hydroxide, water, nitric acid and water. Afterwards, capillary was washed with 20 % (v/v) solution of γ-methacryloxypropyltrimethoxysilane (γ-MAPS) in ethanol. Next, capillary was continuously flushed with solution of 4% (w/v) acrylamide in freshly boiled deionized water with addition the initiator system composed of 0.1 % (w/v) potassium peroxodisulfate and 0.1 % (v/v) tetramethylethylenediamine (TEMED) for 12 hours. Finally, the capillary was rinsed with deionized water for 30 minutes.

Optimization of separation conditions

Based on previously reported separation of tartaric acid [23,24] and other hydroxy acids [25,26], chiral selector based on copper(II) chloride and L-OH-PRO was selected. Contactless conductivity detection [27] was utilized as tartaric acid does not possess any chromophore.

A set of compounds with different acidobasic properties [28]: ß-alanine EACA, L-histidine, and bistris was tested as the BGE counterion providing proper buffering capacity. Best results were achieved regarding peak symmetry response with 100 mM EACA with pH 5.0.

Interestingly, change in migration order was observed for BGEs containing EACA and bistris compared to presence of L-histidine. This was further studied using D-histidine as counterion as well as D-OH-PRO as the ligand. Results are summarized in Table 1. As D-tartaric acid is expected to be present as the minor enantiomer, its migration position as the first is more favorable, therefore the BGE composed of EACA and L-OH-PRO was chosen for further optimization.

Table 1. Migration order of tartaric acid enantiomers depending upon composition of the BGE.

BGE counterion	ligand	first peak	second peak
EACA	L-OH-PRO	D	L
bistris	L-OH-PRO	D	L
L-histidine	L-OH-PRO	L	D
D-histidne	L-OH-PRO	D	L
EACA	D-OH-PRO	L	D
bistris	D-OH-PRO	L	D
L-histidine	D-OH-PRO	L	D
D-histidne	D-OH-PRO	D	L

Concentration of copper(II) (keeping the same 1:2 ratio with L-OH-PRO) was studied regarding the resolution of the enantiomers. Results are shown on Figure 5.

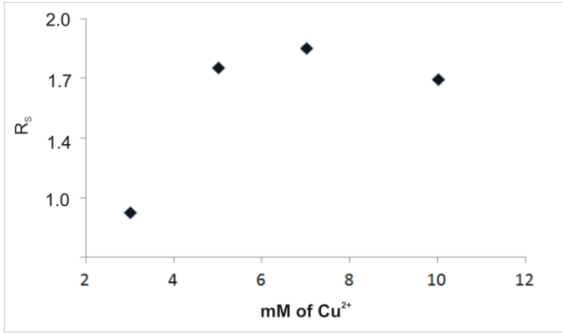

Figure 5. Dependence of resolution on concentration of Cu^{2+} in the BGE. L-OH-PRO was present at ratio 2 : 1 to Cu^{2+}, the BGE contained 100 mM EACA and its pH was adjusted to pH 5 by hydrochloric acid.

Optimal separation conditions were found with 7 mM copper(II) chloride, 14 mM L-OH-PRO, and 100 mM EACA pH 5.0 (adjusted by hydrochloric acid). Separation of a standard racemic mixture of tartaric acid enantiomers is shown on Figure 6.

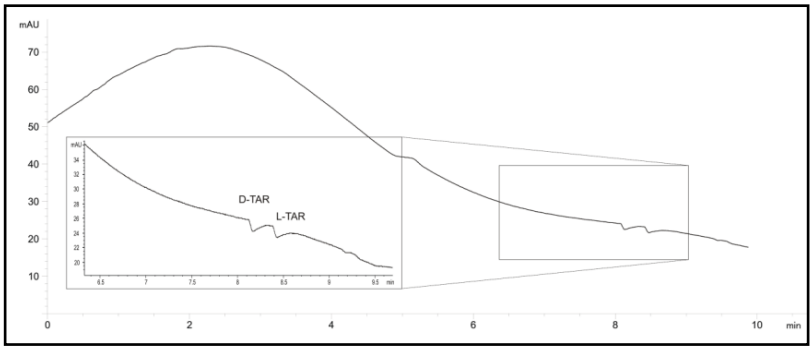

Figure 6. Separation of standard mixture of D,L-tartaric acid, concentration of each enantiomer was 50 μM.

Sample analysis

The developed method was applied on analysis of beverage samples as wine, grape juice, and pharmaceuticals where tartaric acid is present as a counter-ion to the active substance. Figure 7 shows electropherograms of analysis of a white wine sample after 100 times dilution and the same sample spiked with D,L-tartaric acid with an increase of the final concentration of 47 µM. There were no interferences found in the position of the two enantiomers in the samples.

The content of L-tartaric acid in white wine was found as 1.20 ± 0.09 g/l, in red wine 1.9 ± 0.1 g/l and in grape juice 5.3 ± 0.4 g/l. D-tartaric acid was not found in any sample proving no bacterial contamination. The recovery was 104 ± 4%.

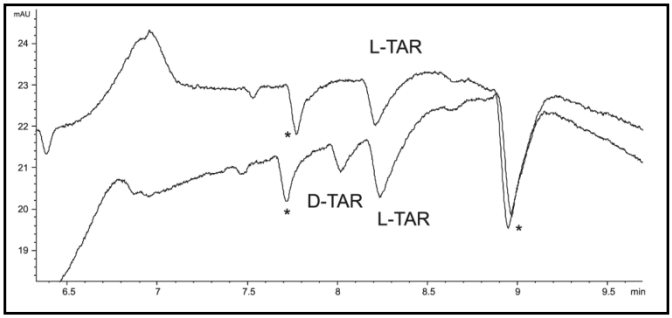

Figure 7. Analysis of 100 times diluted white wine sample (upper trace) and the sample spiked with D,L-tartaric acid (lower trace).

Purity of pharmaceutical substances was assessed in formulations of metoprolol tartrate and zolpidem tartrate. Only L-tartrate was found in the samples, content found in the 0.1 mg/ml samples was as follows: 34 ± 2 µg/ml in metoprolol (corresponds to 95.5 % of the theoretical content assuming 100% purity) and 31 ± 2 µg/ml in zolpidem (corresponds to 94.6 % of the theoretical content).

Porous layer open tubular columns fabrication

Monolithic, or continuous porous stationary phases have attracted significant interest due to their porous properties allowing the use of high flow rates at low backpressures, relatively simple preparation in capillaries with small internal diameters, lack of frits and the ability to control both the physical and chemical properties of the material [29,30]. These properties make them very attractive for PLOT columns.

Photoinitiation using LED oven

Among many approaches for fabrication of PLOT columns, very recently Collins *et al*. [31] have reported use of a device containing several circular arrays of UV light emitting diodes (LED). Capillary is delivered through the centre of a UV chamber to provide equal intensity thus ensuring an evenly distributed porous polymer. Several parameters are expected to influence the resulting polymer and its thickness.

For construction of LED oven 385 nm LEDs "Lumex SSL-LXTO46UV1C" were selected. A scheme of the LED oven consisting of 4 LEDs is shown on Figure 8.

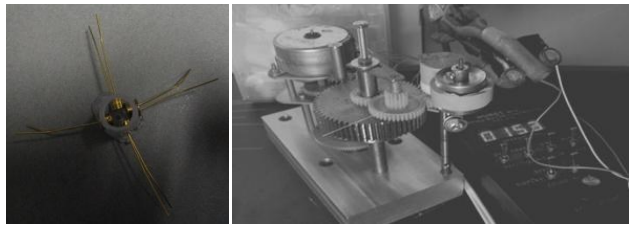

Figure 8. Picture of LED oven before soldering (left) and assembled in the device for capillary delivery equipped with a step-motor.

Polymerization mixture reported before [32] was used for preparation of porous polymers inside UV-transparent Teflon-coated silica capillary: 0.4g ethylene dimethacrylate (EDMA), 0.6 g glycidyl methacrylate (GMA), 1.4 g 1-dodecanol, and 0.93 g cyclohexanol. Bis(2,4,6-trimethylbenzoyl)-phenylphosphineoxide (BAPO) was used as a photoinitator that absorbs light till 440 nm [33].

When BAPO was used at concentration 1 % (m/m) (relative to monomers) a monolith filling whole volume of the capillary was obtained even at fastest delivery speed and lowest power input of the LED oven. Decrease of BAPO content to 0.2 % (m/m) led to formation of highly irregular polymer layer at the capillary wall. Further decrease of BAPO to 0.1% led to largely unrepeatable results possibly explained by increased sensitivity of the polymerization process to impurities and presence of absorbed oxygen over time. Therefore other options of influencing the polymerization kinetics were considered.

Polymerization inhibition by hydroquinone

Inhibition of the polymerization by a radical scavenger was studied. In order to control the amount of the inhibitor and hence control the thickness of the polymer coating on the capillary wall, different content of hydroquinone in the polymerization was examined. Figure 9 shows SEM images obtained from 10 cm long columns from which 5 cm was passed through the LED oven.

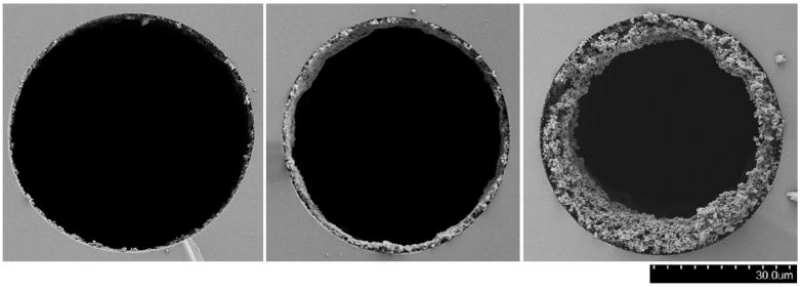

Figure 9. SEM images of columns produced with polymerization mixture containing hydroquinone at concentration 1 mg/g (left), 0.8 mg/g (middle), and 0.5 mg/g (right).

As it was successful to produce PLOT columns with different thickness of the layer (about 1 µm for 1 mg/g of hydroquinone, 3 µm for 0.8 mg/g, and 6-10 µm for 0.5 mg/g), the procedure was applied on capillaries 50 cm in length. However, when longer columns were delivered through the LED oven, inhomogeneity of the thickness distribution was observed along the length of the capillary.

Light attenuation by 4-nitroaniline

To suppress the described effect of light propagation in the capillary an addition of light-absorbing compound was considered. That would limit the amount the light penetrating the polymerization solution deeper inside the capillary and hence increase the chance of formation of the polymer only at the capillary wall.

By varying the amount of BAPO as initiator and 4-nitroaniline as the compound responsible for light attenuation and also by changing capillary movement speed and LED input it was possible to influence the thickness of the porous polymer at the capillary wall. Concentration of 4-nitroaniline was the critical parameter followed by speed of the speed of the capillary through the LED oven responsible for the dosage provided.

However, the previously observed issue with attachment of larger polymer particles leading to inhomogeneity of thickness distribution was still present, although its occurrence was much reduced compared to results obtained with inhibition by hydroquinone. Examples are shown on Figure 10. That could be explained by sedimentation of larger polymer chains or particles in the direction of gravity which corresponds to location of a thicker layer at one side of the capillary wall. For such reasons column-to-column reproducibility was considerably poor and probably length of the prepared column and correspondingly total time of the irradiation/polymerization process would be another factor having an impact on the results. Light attenuation approach was not pursued any further.

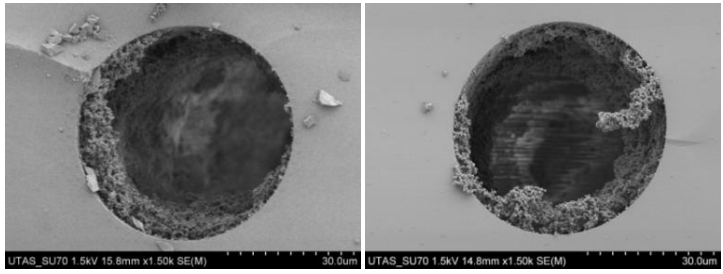

Figure 10. SEM images of cuts obtained with light attenuation by 4-nitroaniline.

Elimination of polymerization kinetics by immiscible liquid

- R. Knob, M.C. Breadmore, R.M. Guijt, J. Petr, M. Macka, *Porous layer open tubular monolith capillary column: switching-off the reaction kinetics as the governing factor in their preparation by using an immiscible liquid controlled polymerization*. RSC Advances, 3, 2013, 24927-24930.

In order to improve the inhomogeneity of the polymer thickness different approaches were investigated that rely on polymerization of whole length of the capillary at the same time. Adsorption of BAPO was on γ-MAPS modified capillary surface was evaluated, however, according to observed results the initiator was partly washed out when capillary was filled with the monomer mixture in the porogenic solvents (1-dodecanol, cyclohexanol). The beginning part of the capillary was left without any polymer attached whereas the end of capillary was filled with the polymer.

A modified experiment was performed based on replacement of the polymerization mixture from the centre of the capillary by a phase without monomers forming the polymer. Several solvents were evaluated, e.g. cyclohexanol, 1-dodecanol, ethylacetate, ethyleneglycol, heptane or hexane. However, such solvents are more or less miscible with polymerization mixture constituents. For such reasons attention was brought to FC-770, a fully fluorinated solvent. Largely different physico-chemical properties of FC-770 from the constituents of the polymerization mixture ensure immiscibility.

The process of replacement of the two liquids was monitored with use of optical microscope and images are shown on Figure 11. Figure 11A shows a boundary between the liquids in γ-MAPS modified capillary, the profile suggest superior wettability of the polymerization mixture (on the left side) over FC-770. Therefore after completely removing polymerization mixture in the capillary with FC-770 with flow rate of 100 μl/h for 3 minutes, only a thin layer of polymerization mixture remains on the capillary wall as shown on Figure 11B.

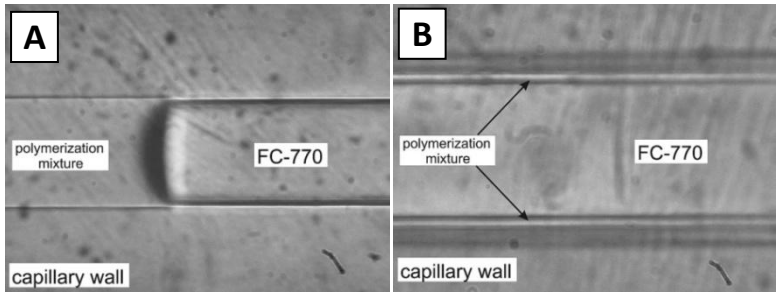

Figure 11. Optical microscope images of γ-MAPS modified capillary filled with polymerization mixture and FC-770. (A) shows a boundary between two liquids, (B) shows a thin layer of polymerization mixture left after replacement with FC-770.

After polymerization using a Shark series high-flux UV LED array with exposure for 1 minute, a homogeneous thin (about 0.25 μm) layer of porous polymer was obtained as shown on SEM images in Figure 12.

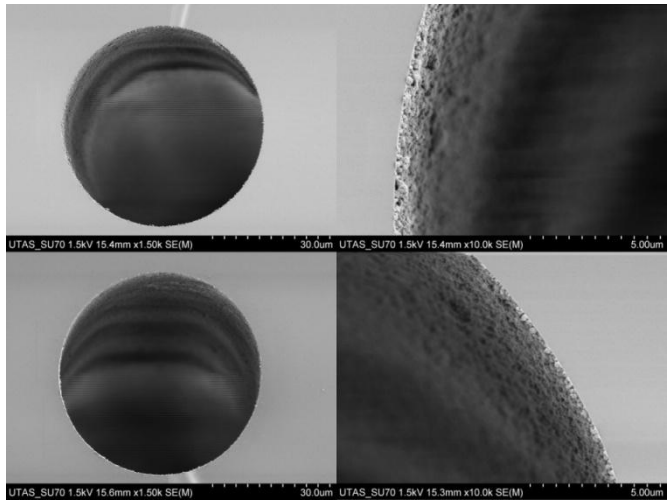

Figure 12. SEM images of two cuts of PLOT modified capillary prepared using replacement by immiscible liquid approach.

Capillary electrochromatography of inorganic anions

The applicability of the column was demonstrated on capillary electrochromatography of selected inorganic anions (chloride, bromide, nitrate) as model analytes.

The capillary was flushed with 0.1 M $NaHSO_3$ for ring-opening of the epoxy groups by treatment at 80˚C for 24 hours [34]. After coupling the PLOT capillary to the uncoated capillary, 1% (m/v) aqueous suspension of Dionex AS18 anion-exchange latex particles was flushed through the capillary at 4 bars for 5 minutes. Next, the capillary was flushed with water and then with the BGE for 10 min each [35].

Figure 13 shows electropherograms of the model mixture using perchlorate Tris-based BGE of pH 8.0. In case of 10 mM BGE, the strongest interactions with the latex nanoparticles modified PLOT column were obtained which allowed iodide to be retained considerably more than the other two ions resulting in a change in selectivity.

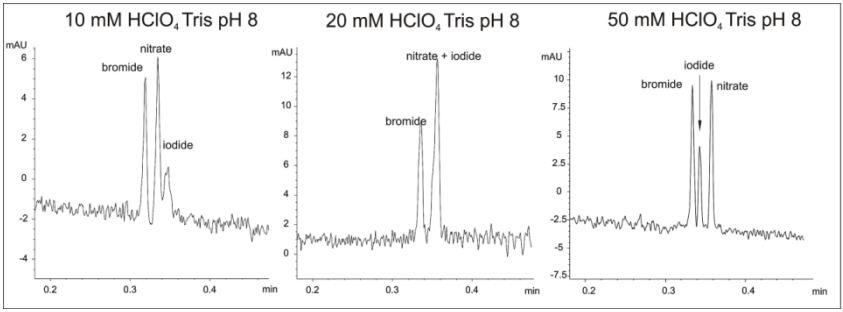

Figure 13. Separation of selected inorganic anions at 0.1 mM using modified anion exchange PLOT column and the BGEs with various elution strength. Voltage 20 kV, injection at short end of the column. Detection at 200 nm.

Increasing the concentration of the BGE increases the elution strength of the electrolyte which reduces the extent of interaction with the PLOT thus the effective mobility is increased. In 20 mM BGE, iodide co-migrates with nitrate (Figure 13, middle), with higher BGE concentration further reducing interaction with the PLOT and a baseline separation is achieved (Figure 13, right).

In comparison, the same separation in an uncoated capillary without modification by the quaternary ammonia latex nanoparticles is shown in Figure 14. Co-migration of bromide and iodide was observed due to the absence of any ion-exchange interactions. The lower and higher concentration of the BGE did not bring any further changes in the separation order as was the same as in the PLOT column.

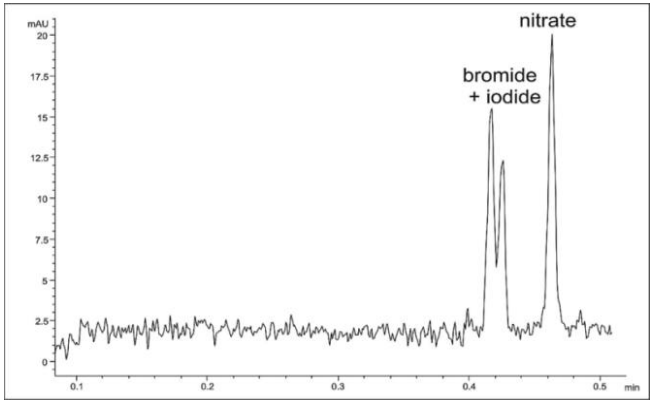

Figure 14. Separation of selected inorganic anions using unmodified silica capillary and the BGE of 10 mM $HClO_4$ Tris pH 8. Conditions are the same as on Figure 13.

Results obtained with latex nanoparticles modified PLOT column were compared with previously reported open tubular column modified with the same nanoparticles [36] regarding effective mobilities of the selected inorganic anions and corresponding capacity factor (*k*'). Table 2 shows the comparison of three columns with different surface modifications and migration parameters.

Table 2. Effective mobilities and capacity factors of bromide, iodide, and nitrate in an uncoated capillary, open tubular [36], and PLOT modified columns using 10 mM $HClO_4$ Tris pH 8.

		bromide	**iodide**	**nitrate**
μ_{eff} **[× 10^{-9} m^2/Vs]**	uncoated	-81.2	-79.6	-75.2
	open tubular	-71.6	-64.7	-66.2
	PLOT	-70.5	-61.7	-65.4
k'	open tubular	0.118	0.187	0.120
	PLOT	0.132	0.225	0.130

Using 10 mM $HClO_4$ Tris pH 8.0 as the BGE, increase of the capacity factor for the PLOT columns compared with the open tubular was 11.5 % for bromide, 20.1 % for iodide, and 8.9 % for nitrate.

CONCLUSION

In conclusion, so far amazing variety of capillary coatings has been developed and reported to use, they are reflected in the theoretical part of the thesis. However, there is no such versatile procedure that can be generally used for every application as different aims, surface properties or effects are desired.

The experimental part of the thesis was focused on separation of compounds with pharmaceutical and physiological relevance using various capillary coatings. Manipulation of migration order was studied by using different coatings that allowed to tune the EOF direction and magnitude, also in chiral separation the use of different chiral selectors allowed to change the migration order.

Several surface modifications were evaluated for analysis of decarboxylated products of *S*-adenosylmethionine as impurities present in a pharmaceutic sample, best results regarding resolution of the diastereoisomers were achieved using the background electrolyte based on barium borate. It was possible to determine their content less than 0.1 % in 40 mg/ml sample.

Polyacrylamide covalent coatings was used for separation of tartaric acid enantiomers using contactless conductivity detection. Chiral separation was achieved using a complex of copper(II) and *trans*-4-hydroxy-L-proline as a chiral selector. Several counter-ions were tested and the possibility of reversal of migration order of tartaric acid enantiomers was studied as it is advantageous when the minor enantiomer migrates as the first for quantitation. The method was applied on analysis of wine, grape juice, and pharmaceutical formulations, however, no D-tartaric acid was found.

A new approach for fabrication of thin layer PLOT column based on use of immiscible liquid was developed. Together with increased capacity in comparison to open tubular columns, such columns offer fast rinsing times and has no requirements on pumps. The procedure allows preparing the homogeneous coating as the stationary phase for capillary electrochromatography. The applicability of the prepared columns was demonstrated on the separation of three selected inorganic anions (bromide, iodide, and nitrate), that was achieved within 22 seconds by injection on the shorter end of the capillary. By changing the elution strength of the BGE it was possible to change the migration order of the analytes.

The developed procedure for fabrication of such columns could be possibly applied for other applications than only in capillary electrophoresis that would benefit from the increased surface area and high permeability. Compatibility with other polymerization mixtures and initiators, possibility of production thicker coatings or deposition of multiple layers will be certainly studied in the near future.

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CURRICULUM VITAE

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Education:

2005 - 2008	Bachelor study of chemistry, Faculty of Science, Palacký University in Olomouc, defense of bachelor thesis: *Separation of organic acids by capillary electrophoresis with contactless conductivity detection*
2008 - 2010	Master study of analytical chemistry, Faculty of Science, Palacký University in Olomouc, defense of diploma thesis: *Polypyrrole coated capillaries for capillary electrophoresis*
2012	Post-graduate doctoral degree (RNDr.), Faculty of Science, Palacký University in Olomouc, defense of rigorous thesis: *Analýza vybraných bromovaných fenolů pomocí on-line spojení kapilární izotachoforézy s kapilární zónovou elektroforézou* (Analysis of selected brominated phenols by on-line coupled capillary isotachophoresis and capillary zone electrophoresis)
2010 - till now	Ph.D. study of analytical chemistry, Faculty of Science, Palacký University in Olomouc, topic: Study on capillary coatings for capillary electrophoresis

Research / Work Experience:

2008	Research stay, Chair of Environmental Chemistry and Ecotoxicology (supervisor: prof. Hartmut Frank), University of Bayreuth, Germany, February – April.
2009 - 2011	Vocational work, TEVA Czech Industries, Opava-Komárov, Solid-state R&D (supervisors: Dr. Aleš Gavenda and Dr. Aleš Husek), five visits, 4 months in total.
2009	Research stay, Department of Analytical Chemistry (supervisor: Ass. prof. Jozef Marák), Faculty of Natural Science, Comenius University in Bratislava, Slovakia, May – June.
2010	Research stay, Dipartimento di Chimica (supervisor: prof. Pier Giorgio Righetti), Politecnico di Milano, Italy, February – May.
2011 - 2012	Honorary Research Associate at the Australian Centre for Research on Separation Science and School of Chemistry, University of Tasmania, Hobart, Australia, September - May, supervisor: prof. Mirek Macka.
2012 - 2013	Research work, Department of Forensic Medicine and Medical Law, Faculty of Medicine, Palacký University in Olomouc (supervisor: Ass. prof. Peter Ondra), October - June.
2013	Research stay, KIST Europe (supervisor: prof. Andreas Manz), Saarbrücken, Germany, April – May.
2013	Research stay, Australian Centre for Research on Separation Science, University of Tasmania, Hobart, Australia (supervisor: prof. Michael Breadmore), November - December.
2014	Research stay, KIST Europe (supervisor: prof. Andreas Manz), Saarbrücken, Germany, March.

Employment / Projects:

2010 - 2014	Student grant competition, IGA Palacký University in Olomouc, member of the team
2011 - 2014	Kontakt LH11018, *Cyclofructans and their derivatives as new chiral selectors for liquid chromatography and capillary electrophoresis*, member of the team
2012	University Development Fund *(FRVŠ), New laboratory exercises utilizing non-aqueous solvents*, principal investigator
2012 – 2014	IGA Ministry of Health of the Czech Republic NT1393-3/2012, *Diagnostics of intoxication by designer drugs*, member of the team
2012 - 2014	Operational Program Education for Competitiveness CZ.1.07/2.3.00/20.0018, *Development of human resources for excellence in research in nanotechnology in analytical chemistry*, member of the team
2012 - 2014	Operational Program Education for Competitiveness CZ.1.07/2.4.00/17.0065, *Partnership and networks for an excellence in chemistry*, member of the team
2012 - 2014	Operational Program Education for Competitiveness CZ.1.07/2.4.00/31.0006, *Development of research and education in chemistry and medicine of burn injuries*, member of the team
2013	University Development Fund *(FRVŠ), New laboratory exercises of food analysis by capillary electrophoresis*, principal investigator

Teaching:

2011	Laboratory course of analytical chemistry for 2nd year students of bachelor degree, 1 semester
2012	Laboratory course of analytical and environmental chemistry for 2nd year students of bachelor degree, 1 semester

Publication activity:

[1] V. Maier, J. Petr, R. Knob, J. Horáková, J. Ševčík: *Electrokinetic partial filling technique as a powerful tool for enantiomeric separation of D,L-lactic acid by capillary electrophoresis with contactless conductivity detection*. Electrophoresis **28** (2007) 1815.

[2] J. Znaleziona, J. Petr, V. Maier, R. Knob, J. Horáková, D. Smetanová, J. Ševčík: *Capillary electrophoresis as a verification tool for immunochemical drug screening*. Biomed. Pap. Med. Fac. Univ. Palacky Olomouc **151** (2007) 31.

[3] O. Ryparová, J. Petr, M. Kowalska, J. Znaleziona, R. Knob, V. Maier, I. Frébort, J. Ševčík*: Analýza mikroorganismů metodou kapilární elektroforézy*. Chem. Listy **102** (2008) 1121.

[4] J. Petr, K. Vítková, J. Znaleziona, V. Maier, V. Ranc, R. Knob, J. Ševčík: *Determination of some phenolic acids in Majorana hortensis by capillary electrophoresis with on-line electrokinetic preconcentration* J. Agric. Food Chem. **56** (2008) 3940.

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[7] R. Knob, R. Čabala, S. Gerstmann, H. Frank: *Polypyrrole-coated and polysulfate-modified CE capillaries*. Chromatographia **69** (2009) 1431.

[8] R. Knob, J. Marák, A. Staňová, V. Maier, D. Kaniansky, J. Ševčík: *Determination of brominated phenols in water samples by on-line coupled isotachophoresis with capillary zone electrophoresis.* J. Chromatogr. A **1217** (2010) 3446.

[9] R. Sebastiano, R. Knob, A. Citterio, P.G. Righetti: *Analysis of trace degradation products (decarboxylated diastereoisomers) of S-Adenosylmethionine by electrophoresis in capillaries with cationic coatings (N-methylpolyvinylpyridinium or divalent barium).* Electrophoresis **31** (2010) 3592.

[10] R. Knob, V. Maier, J. Petr, V. Ranc, J. Ševčík: *On-line preconcentration of perfluorooctanoic acid and perfluorooctanesulfonic acid by non-aqueous capillary electrophoresis*. Electrophoresis **33** (2012) 2159.

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[12] R. Knob, J. Petr, J. Ševčík, V. Maier: *Enantioseparation of tartaric acid by ligand-exchange capillary electrophoresis using contactless conductivity detection.* Journal of Separation Science **36** (2013) 3426.

[13] R. Knob, M. Breadmore, R. Guijt, J. Petr, M. Macka: *Porous layer open tubular monolith capillary column: switching-off the reaction kinetics as the governing factor in their preparation by using an immiscible liquid-controlled polymerization.* RSC Advances **3** (2013) 24927.

Book chapters:

R. Knob, M. Macka, P.R. Haddad: *Capillary Electrochromatography* in *Encyclopedia of Analytical Science*, Third edition, Elsevier 2013.

Conference oral presentations during Ph.D. study:

[1] R. Knob, M.C. Breadmore, R. Guijt, J. Petr, M. Macka: *Control of the polymerization towards porous layer open tubular capillary columns.* CECE 2012, 1. - 2. November 2012, Brno, Czech Republic.

[2] R. Knob: *Analýza nečistot farmaceutických substancí*. Medicína a chemie popáleninových stavů, 27. Februrary 2014, Ostrava, Czech Republic.

Conference poster presentations during Ph.D. study:

[1] R. Knob, V. Maier, J. Petr, V. Ranc, J. Ševčík: *First time capillary electrophoretic separation of perfluorooctanoic acid and perfluorooctanesulfonic acid in non-aqueous media*, Nordic Separation Science Society 6th Conference, 24. – 27. 8. 2011, Riga, Latvia.

[2] R. Knob, V. Maier, J. Petr, V. Ranc, J. Ševčík: *Possibilities of on-line preconcentration of perfluorooctanoic acid and perfluorooctanesulfonic acid by non-aqueous capillary electrophoresis*, 18th International Symposium on Electro- and Liquid Phase-separation Techniques (ITP 2011), Tbilisi, Georgia, 28. - 31. August 2011.

[3] R. Knob, V. Maier, J. Petr, V. Ranc, J. Ševčík: *Analysis of perfluorooctanoic acid and perfluorooctanesulfonic acid by non-aqueous capillary*

electrophoresis, 11th Asia Pacific International Symposium on Microscale Separations and Analysis (APCE 2011), Hobart, Australia, 27. – 30. November 2011.

[4] R. Knob, M. Breadmore, R. Guijt, M. Macka: *PLOT monolith modified capillaries for capillary electrochromatography*, 19th RACI Research And Development Topics Conference in Analytical and Environmental Chemistry, Melbourne, Australia, 7.-9. December 2011.

[5] R. Knob, M. Breadmore, R. Guijt, M. Macka: *PLOT monolith capillary columns for on-line preconcentration in capillary electrophoresis*, 36th Internation Symposium on Capillary Electrochromatography (ISCC 2012), Riva del Garda, Italy, 27. May – 1. June 2012.

[6] R. Knob, M. Breadmore, R. Guijt, M. Macka: *Preparation of PLOT monolith columns*, Advances in Chromatography and Electrophoresis & Chiranal 2012, Olomouc, Czech Republic, 11. – 14. June 2012.

[7] R. Knob, A. Přibylka, P. Ginterová, P. Ondra, I. Válka, V. Maier: *CE-ESI-MS/MS analysis of psychotropic drugs, 29th International Symposium on Chromatography, Torun, Poland, 9. - 13. September 2012.*

[8] R. Knob, P. Ginterová, I. Válka, P. Ondra, V. Maier: *Derivatization of selected designer drugs for GC-MS analysis*. Analytické metódy a zdravie človeka, Rajecké Teplice, Slovakia, 24. – 27. June 2013.

[9] R. Knob, J. Petr, J. Ševčík, V. Maier: *Enantioseparation of tartaric acid by ligand-exchange capillary electrophoresis and isotachophoresis.* 20th International Symposium on Electro- and Liquid Phase-Separation Techniques, ITP 2013, 6. – 9. October 2013, Tenerife, Spain.

[10] R. Knob, J. Křenková, J. Petr, F. Foret: *Porous layer open tubular columns with immobilized trypsin for protein digestion.* CECE Junior 2013, 12. - 13. November 2013, Brno, Czech Republic.

[11] R. Knob, V. Maier, M. Švidrnoch, J. Ševčík, J. Petr: *Separation of selected designer drugs by porous layer open-tubular capillary electrochromatography.* 40th International Symposium on High Performance Liquid Phase Separations and Related Techniques, HPLC 2013, 18. – 21. November 2013, Hobart, Australia.

Other activities:

Popularization activities of Department of Analytical Chemistry at Faculty of Science, Palacký University in Olomouc

Member of organizing committee of Chiranal conferences (2006 - 2014)

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