

Article

# Monolithic Silica Capillary Columns with Improved Retention and Selectivity for Amino Acids

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**Abstract:** A strategy for the preparation of silica-based monolithic capillary columns (150 × 0.1 mm) with high selectivity to amino acids is presented. The zwitterionic columns were prepared by coating the silica monolith with [2-(methacryloyloxy)ethyl]-dimethyl-(3-sulfopropyl)-ammonium hydroxide via 3-(trimethoxysilyl)propyl methacrylate. The columns were evaluated under isocratic conditions in hydrophilic interaction liquid chromatography. The best separation of amino acids was obtained on the monolithic column prepared by a stepwise modification procedure where the modification step was repeated four times. The mixture of fifteen amino acids was separated within 13 min using the mobile phase consisting of 75% acetonitrile and 25% 5 mmol/L ammonium acetate at pH 4.5.

**Keywords:** HILIC; amino acids; silica monolithic column

## 1. Introduction

Analysis of amino acids has become an important task in different areas of life sciences such as foodomics, proteomics, metabolomics, and biopharmaceuticals, where accurate, precise, and robust amino acid analyses are required [1–5]. Liquid chromatography (LC) is widely utilized to determine the content of amino acids in samples. These are usually determined after the chemical derivatization, which improves their chromatographic behavior or increases their detection sensitivity by ultra violet (UV), fluorescence, or mass spectrometric (MS) detection [6]. LC analysis of native amino acids has become ever more popular due to the availability of highly-sensitive MS detection. The gradient or multi-gradient elution conditions are used in hydrophilic interaction chromatography (HILIC) or reversed-phase chromatography (RPLC) to resolve complex mixtures of native amino acids or their derivatives [1–5,7]. The weakness of this approach is the need for re-equilibration of the system to initial conditions, which increases the total time of analysis. If a large number of samples is analyzed, avoiding the gradient elution could be of significant benefit, because of increased sample throughput and reduced solvent consumption.

Capillary liquid chromatography is a highly popular and widespread separation technique in laboratories working in the research areas of pharmacology, proteomics, and metabolomics. Moreover, many reviews published in recent years summarize trends in the design and evaluation of instruments suitable for capillary and nano-liquid chromatography [8–13].

The most important part of the LC system is the chromatographic column, which enables successful resolution of complex samples containing a large number of components. The particle-packed capillary columns available on the market are packed with the same sorbents as columns of analytical dimensions. Thus, a wide range of packed capillary columns is available. On the other hand, the supply of monolithic capillary columns is limited to columns suitable for RPLC, such as the polymer-based ProSwift™ monolithic LC columns, supplied by Thermo Scientific™ (Waltham, MA, USA), the silica-based monolithic columns Chromolith® CapRod®

capillary LC columns, supplied by Merck KGaA (Darmstadt, Germany), Onyx™ Monolithic C18 column supplied by Phenomenex (Torrance, CA, USA), and, last but not least, a company offering silica-based monolithic columns is GL Sciences Inc. (Tokyo, Japan) with MonoCap series of columns suitable for RPLC, HILIC, and strong-cation-exchange chromatography. Along with an ever-expanding supply of monolithic columns, new monolithic materials which are suitable for highly-specialized separations are being prepared in laboratories around the world [14–18].

However, the conventional RPLC columns, as well as other recently introduced, specialized columns, are not suitable for the isocratic LC separation of underivatized amino acids. Therefore, the presented study is focused on the preparation of zwitterionic silica-based monolithic capillary columns suitable for the isocratic HILIC separation of native amino acids.

The concept for the synthesis of silica-based monolithic columns was published by Nakanishi and coworkers in the early 1990s [19,20]. The first silica monoliths were prepared by acidic hydrolysis of tetramethoxysilane (TMOS), or mixture of TMOS and methyltrimethoxysilane. An appropriate stationary phase was obtained by a chemical postmodification procedure [21]. Later, various alkyltrialkoxysilanes and their mixtures with alkoxysilanes or organic monomers were utilized to synthesize silica-based monolithic capillary columns [22].

Our monoliths were prepared by acidic hydrolysis of TMOS in the presence of polyethylene glycol and urea. The plain silica monolith was then modified by 3-trimethoxysilylpropyl methacrylate ( $\gamma$ -MAPS) to introduce vinyl groups to the monolithic silica surface. Thermally-initiated free radical polymerization was applied to create a polymer coating of zwitterionic [2-(methacryloyloxy)ethyl]-dimethyl-(3-sulfopropyl)-ammonium hydroxide (MSA) on the silica surface. MSA is a commercially-available substance which is frequently used for the synthesis of monolithic columns. Preparation and LC characterization of the first polymer-based monoliths containing MSA suitable for ion-exchange chromatography of proteins and purification of peptides was published by Viklund et al. [23]. MSA capillary monoliths were prepared a few years later, and evaluated under HILIC conditions for separation of polar compounds such as neutral acrylamides, benzoic acid derivatives, and nucleobases [24]. MSA monoliths show a multimodal retention mechanism due to the nature of the MSA monomer. Urban et al. [25] investigated the transition between RPLC and HILIC mechanisms on monoliths prepared from a mixture of MSA and ethylene dimethacrylate.

Silica monolithic capillary columns prepared from TMOS and modified to a sulfobetaine type zwitterionic stationary phase were used for site-specific glycosylation analysis by Wohlgemuth et al. [26] and for separation of nucleobases and nucleosides under isocratic and gradient HILIC conditions [27]. Lin et al. utilized MSA as a part of the polymerization mixture for the preparation of monolithic capillaries by a “one-pot” approach. Benzoic acid derivatives, phenols, purines and pyrimidines, and nucleosides were used to evaluate the prepared columns under HILIC conditions [28].

## 2. Materials and Methods

### 2.1. Chemicals and Reagents

Ammonium acetate, acetic acid, acetonitrile (ACN, LC/MS-grade), [2-(methacryloyloxy)ethyl]-dimethyl-(3-sulfopropyl)-ammonium hydroxide, toluene, absolute ethanol, methanol, sodium hydroxide, hydrochloric acid (p.a.), azobisisobutyronitrile (AIBN), xylene mixture of isomers (Product #: 95690), 3-trimethoxysilylpropyl methacrylate ( $\gamma$ -MAPS), and all standard compounds used were purchased from Sigma-Aldrich (Austria). Water purified with a Milli-Q A10 Gradient (Millipore, Burlington, MA, USA) was used in the experiments.

## 2.2. Instrumentation

The equipment used for LC-UV consisted of a syringe pump (100 DM with D-series controller, Teledyne Isco, Lincoln, NE, USA), an electrically actuated E90-220 injection valve with a 60 nL or 500 nL inner loop (Valco, Houston, TX, USA), and a T-splitter with a restrictor (fused silica capillary 0.025 mm i.d.  $\times$  150 mm length). The monolithic column outlet was connected to a Spectra 100 UV-Vis detector (Thermo Separation Products, Waltham, MA, USA) via a fused silica capillary of dimensions 0.035 mm i.d.  $\times$  110 mm, with a bubble cell optical window (0.110 mm i.d.) made by controlled etching. The geometrical volume of this connecting capillary was 0.106  $\mu$ L. UV-detection was performed at 210 nm. Data were collected and processed using DataApex Clarity 5.02 software.

## 2.3. Preparation of Monolithic Capillary Columns

Plain monolithic silica-based capillary columns were prepared by acidic hydrolysis of TMOS in the presence of polyethylene glycol (molar mass of 10,000) and urea, and modified by 20% (*v/v*) of  $\gamma$ -MAPS in 95% (*v/v*) ethanol containing 5% (*v/v*) of acetic acid and water (1:1). The preparation protocol was outlined in our previous study [29]. Columns were flushed with absolute ethanol, dried under a stream of nitrogen at 25 °C for 6 h, and then used for further modification to the zwitterionic stationary phase.

A mixture of methanol (30%, *v/v*) and xylenes (70%, *v/v*) was employed as the solvent in the grafting mixture. AIBN at concentration of 3 wt.% proportionally to monomer MSA (60 mg/mL) was used to initiate the polymerization reaction. The MSA coating was created on the silica monolith as follows: The capillary column modified by  $\gamma$ -MAPS was flushed by a solution of MSA at 25 °C and a flow rate of 0.5  $\mu$ L/min for 20 min. The ends of the column were sealed by the septum and static modification proceeded at 80 °C for 1.5 h. Then, the column was extensively flushed by a mixture of methanol and xylenes, by pure methanol, and finally by a mixture of ACN and the acetate buffer to remove remaining MSA homopolymers out of the column. The column was shortened to 150 mm and evaluated under HILIC conditions using toluene and uracil as a sample. The modification procedure mentioned above was repeated (1–4 times) to prepare monolithic capillaries with improved retention of analytes and selectivity of the stationary phase as discussed below. The capillary column was extensively flushed by a mixture of 70% ACN/30% water, dry methanol, and dried under the stream of nitrogen at 25 °C for 3 h before the creation of the next MSA coating layer on the monolithic stationary phase.

## 2.4. Preparation of Mobile Phases and Sample Solutions

The premixed mobile phase consisted of 70% ACN and 30% ammonium acetate buffer pH 4.5 at the concentration of 5 or 25 mmol/L.

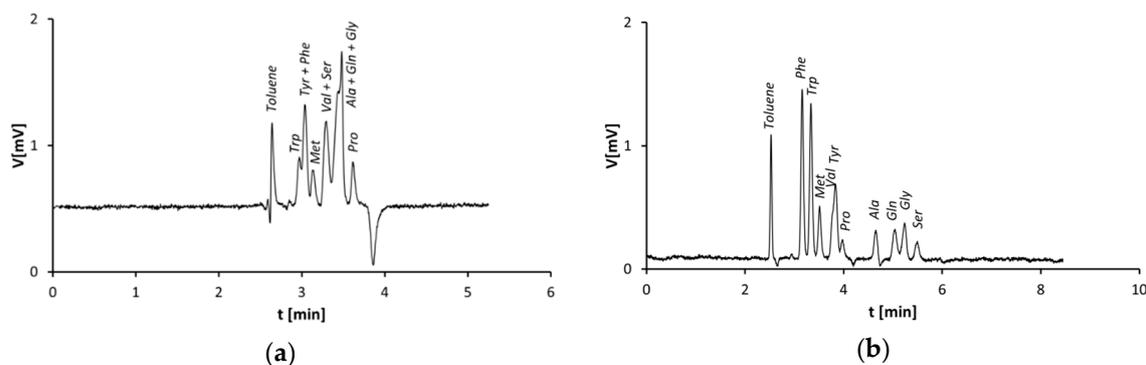
The sample solution was prepared in 70% ACN and 30% 5 mmol/L ammonium acetate at pH 4.5. Because of the UV-detection and nature of analytes, the sample mixture for the evaluation of the synthesized columns contained 0.5  $\mu$ L/mL toluene and 0.5 mg/mL phenylalanine, tryptophan, and histidine. The concentration of other amino acids in the sample mixture was 1 mg/mL of each.

## 3. Results and Discussion

### 3.1. Separation of Amino Acids on Monolithic Silica Capillary Columns—Plain Silica vs. Zwitterionic Stationary Phase

Closely related structures of amino acids require highly selective stationary phases to achieve successful chromatographic separation under isocratic conditions. Figure 1a shows that a non-functionalized silica monolithic column has poor separation selectivity to amino acids under HILIC conditions. A better selectivity for amino acids was achieved on the zwitterionic column (Figure 1b) prepared by the modification procedure as described in [27], where a solution of MSA monomer at a concentration of 15 mg/mL and AIBN at a concentration of 0.45 mg/mL in methanol (30%, *v/v*) and xylenes (70%, *v/v*) was used for modification of the silica monolith. The reaction

proceeded at 80 °C for 3 h. The retention of all amino acids was slightly improved compared to plain silica monoliths, although the pair of valine/tyrosine was not resolved under the aforementioned conditions. Nevertheless, the analysis was fast: the total analysis time did not exceed 6 min; see Figure 1b. On the other hand, the quality of separation was still not comparable to the previously published results for the gradient separation of amino acids on commercial particle packed analytical columns [30,31].



**Figure 1.** HILIC separation of ten amino acids. (a) plain silica monolithic capillary column; (b) MSA monolithic capillary column prepared according to the literature [27]. Operating conditions: columns 150 × 0.1 mm; mobile phase: 75% ACN, 25% 25 mmol/L ammonium acetate at pH 4.5; flow rate 500 nL/min; UV detection at 210 nm.

### 3.2. Capillary Columns Modified by the Stepwise Modification Procedure

To improve the retention and selectivity of the zwitterionic stationary phase, a technique involving the creation of multiple polymer coating layers on the silica monolith was utilized. Compared to the previously published preparation method [27], the solution containing four times higher concentration of MSA (60 mg/mL) was used to modify silica monolithic columns. Each coating was created by flushing of the capillary column by MSA solution for 1.5 h; see Section 2.3 for details. The quality of the resulting stationary phase created on the monolithic silica surface was evaluated through the retention of uracil. Although uracil is not strongly retained under the elution conditions employed. Rather, it serves as an example to illustrate how the retention of it indeed increases as a function of each modification. The variation in retention of uracil between individual modification steps is low, as reflected by the values of retention factor of uracil,  $k_{uracil}$ , listed in Table 1. The monolithic column containing four polymer coating layers of MSA shows a 3.5 times greater value of  $k_{uracil}$  than the column after the first modification step (1 coating layer) and 11-fold greater value of  $k_{uracil}$  than plain silica monolithic column.

**Table 1.** The total porosity, retention factors, and efficiency of prepared monolithic columns.

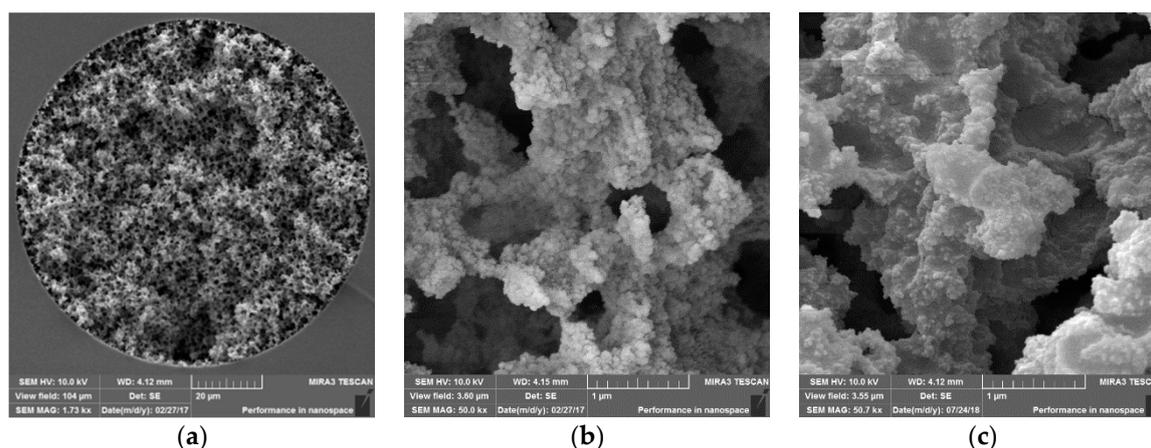
Monolith	Total Porosity $\epsilon_T$	$k_{uracil}$	$N_{uracil}$
Silica	0.945	0.04	180,200
1st modification	0.916	0.13	176,020
2nd modification	0.836	0.21	169,170
3rd modification	0.739	0.31	155,720
4th modification	0.669	0.45	146,870

$k_{uracil}$ —retention factor of uracil,  $N_{uracil}$ —number of theoretical plates per meter.

Data also shows that the retention factor of uracil increases with an increasing number of polymer coating layers created on the monolithic silica surface, while the column efficiency expressed as a plate count per meter decreases in the same manner. The plain silica monolithic capillary column showed efficiency of 180,200 theoretical plates/m, but the first MSA coating decreased this number to 176,020

theoretical plates/m. The column modified by four coating layers of MSA showed reduced efficiency of 146,870 theoretical plates/m, which corresponds to the theoretical plate height of 6.8  $\mu\text{m}$ .

The total porosity of the column,  $\varepsilon_T$ , was calculated as the ratio of the hold-up volume of the column reduced by a volume of the capillary connecting the column outlet with a UV detection cell, and the geometric volume of the empty fused silica capillary used to prepare the column. The volume of capillary connecting the column and the detector cell cannot be neglected with respect to the geometrical volume of the column (0.11  $\mu\text{L}$  vs. 1.18  $\mu\text{L}$ ); see Section 2.2. The hold-up volume of the column was obtained from the volumetric flow rate of the mobile phase (75% *v/v* ACN in 25 mmol/L ammonium acetate at pH 4.5), and the column hold-up time  $t_0$  determined using toluene.  $\varepsilon_T$  reached a value of 0.95 for the plain silica monolithic column. The first polymer coating layer created on the silica monolith reduced the value of  $\varepsilon_T$  by 3%, but the next modification by MSA caused a decrease in the column porosity by approximately 9–11%; see Table 1. The  $k_{\text{uracil}}$  increased simultaneously with the reduced porosity of the monolithic column. Further modification was not performed due to high backpressure of the prepared column. Figure 2a shows a cross section of the prepared plain silica monolithic column, which was further modified by a MSA coating. Comparison of the plain and the MSA modified monoliths is depicted in Figure 2b,c. Figure 2b shows the detail of the unmodified silica with the bi-modal structure typical for the silica monolith. The structure of the monolith significantly changed when the modification procedure was repeated four times (Figure 2c). The nanopores are covered by a polymer coating of MSA, and the size of the through pores is lowered; changes in values of  $\varepsilon_T$  and  $k_{\text{uracil}}$  (Table 1) reflect these findings.

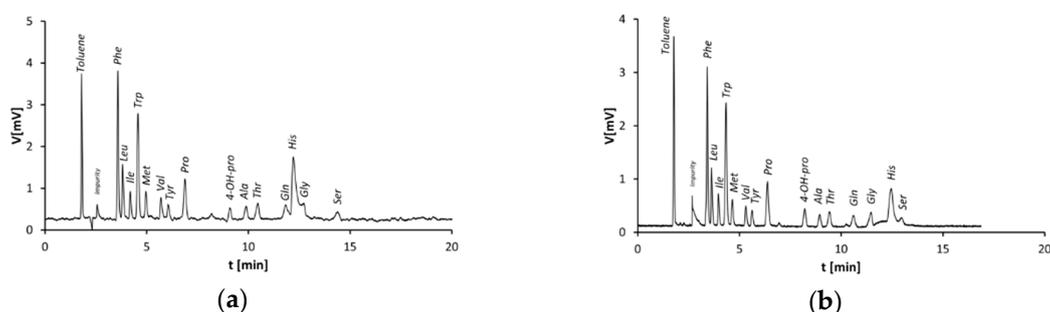


**Figure 2.** Scanning electron microphotographs of prepared monolithic columns. (a) detail of the plain silica monolithic capillary column; (b) unmodified monolithic silica skeleton; (c) monolithic silica skeleton modified by four coating layers of MSA.

Figure 3a confirms that the MSA monolithic capillary column with four polymer coating layers of MSA is suitable for efficient separation of the mixture containing fifteen amino acids under isocratic HILIC conditions. Amino acids were eluted from the column within 15 min when 25 mmol/L ammonium acetate (pH 4.5) was used as a part of the mobile phase. The corresponding retention factors are listed in Table 2. Amino acids with hydrophobic side chains were the first to elute from the column according to the decreasing hydrophobicity. The column is able to separate compounds such as leucine and isoleucine differing in position of the methylene group, as was proven before for nucleosides [27]. In addition, proline and 4-hydroxy proline differing in the hydroxyl group were also well separated on this column.

Buffer concentration in the mobile phase affects the retention and chromatographic resolution of amino acids. A higher concentration of ammonium acetate in the mobile phase caused an increase in the retention of all amino acids except for positively charged histidine; see Table 2 and Figure 3. Histidine also co-eluted with glycine and glutamine when 25 mmol/L ammonium acetate was used

as the aqueous part of the mobile phase (Figure 3a). Baseline separation of glutamine and glycine was obtained using the mobile phase containing five times lower concentration of ammonium acetate (Figure 3b) when the retention time of histidine was shifted to higher values.



**Figure 3.** HILIC separation of fifteen amino acids on MSA monolithic capillary column. Operating conditions: column  $150 \times 0.1$  mm; mobile phase: (a) 75% ACN, 25% 25 mmol/L ammonium acetate at pH 4.5; (b) 75% ACN, 25% 5 mmol/L ammonium acetate at pH 4.5; flow rate 500 nL/min; UV detection at 210 nm.

**Table 2.** Effect of buffer concentration on retention factors of amino acids.

Amino Acid	$k_5$ *	$k_{25}$ **
Phenylalanine	0.92	0.99
Leucine	1.04	1.12
Isoleucine	1.23	1.33
Tryptophan	1.44	1.54
Methionine	1.61	1.76
Valine	1.98	2.17
Tyrosine	2.13	2.37
Proline	2.56	2.83
4-Hydroxy proline	3.59	4.06
Alanine	4.01	4.51
Threonine	4.30	4.82
Glutamine	4.97	5.58
Histidine	6.03	5.79
Glycine	5.46	6.07
Serine	6.29	7.00

\* Retention factors  $k_5$  in mobile phase 75% ACN, 25% 5 mmol/L ammonium acetate at pH 4.5. \*\* Retention factors  $k_{25}$  in mobile phase 75% ACN, 25% 25 mmol/L ammonium acetate at pH 4.5.

#### 4. Conclusions

The capillary monolithic columns prepared by the stepwise modification procedure enabled the separation of fifteen amino acids under isocratic HILIC conditions. This is advantageous when a large number of samples are to be processed, e.g., in the areas of proteomics, metabolomics, or foodomics. The total analysis time was reduced compared to the gradient elution where the re-equilibration time of the column increases the overall time required for analysis. The zwitterionic columns prepared by the stepwise modification procedure using the solution of the MSA monomer allowed a better separation of amino acids than the plain silica monolithic column, as well as the column with one polymer coating layer of MSA. Concentration of the buffer in the mobile phase makes it possible to manipulate the retention characteristics of native amino acids, i.e., a lower buffer concentration in the mobile phase caused an increase in the chromatographic resolution of amino acids such as glutamine, glycine, and histidine.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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