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Article

Reversed-Phase Liquid Chromatography Methods Based on C_{18} Polymer Monoliths for the Determination of Urinary Myoglobin

Renan Vitek,[a] Fernando H. do Nascimento[b] and Jorge C. Masini *,[b]

[a]*Instituto Federal de Educação Ciência e Tecnologia de Mato Grosso, 78043-409 Cuiabá-MT, Brazil*

[b]*Departamento de Química Fundamental, Instituto de Química, Universidade de São Paulo, Av. Prof. Lineu Pestes, 748, 05508-000 São Paulo-SP, Brazil*

This study describes reversed-phase liquid chromatography (RPLC) methods to quantify urinary myoglobin using polymer monolithic columns produced by copolymerization of stearyl methacrylate (SMA) and ethylene glycol dimethacrylate (EDMA). The columns were prepared in the coffins of 1.5 mm internal diameter (i.d.) ethylene tetrafluoroethylene (ETFE) tubing for use in sequential injection chromatography (SIC) and solid phase extraction (SPE), and inside 1.0 mm i.d. Silcosteel® tube for use in narrow-bore liquid chromatography. The monoliths inside the ETFE were produced via UV, whereas thermal polymerization formed the monoliths inside the Silcosteel® tube. The separation of carbonic anhydrase, lysozyme, and myoglobin was demonstrated because they may occur simultaneously in urine samples. Quantification was undertaken by external calibration, and the accuracy was evaluated by the spiking/recovery strategy. The methods exhibited linearity from 5.0 to 60 μg mL^{-1} (SIC), 2.5 to 50 μg mL^{-1} (high-performance liquid chromatography (HPLC)), and 1.0 to 7.5 μg mL^{-1} for an SPE-HPLC method. The lowest limits of detection and quantification were 0.13 and 0.43 μg L^{-1}, respectively, obtained after concentrating myoglobin by SPE. Recoveries ranged from 98 to 105%. The low cost, simplicity, reusability, and analytical features provided by these polymeric stationary phases make them affordable alternatives to routine analyses of urinary myoglobin.

Keywords: protein separation, narrow-bore chromatography, monolithic columns, sample preparation, flow analysis, solid phase extraction

Introduction

Myoglobin is a heme protein responsible for carrying oxygen to cells. It may be present in urine at low concentrations in healthy people and has been used as a marker of oxidative stress.[1] High concentrations can cause some disturbs, such as nephrotoxicity.[2] Urinary myoglobin is an indicator of many diseases, such as myocardial infarction,[3] and the increase above the normal range has been related to prostate cancer.[4] Its concentration increases within intense exercise practice.[5]

There are many methods to quantify myoglobin in urine, including electrochemical analysis,[6] chromatography,[5,7] and immunosorbent assays (ELISA).[8] Liquid chromatographic (LC) methods have been proposed since the 1980s[9] as a noninvasive alternative using size exclusion, anion exchange, hydrophobic interaction and reversed-phase separation modes.[9-12] However, these chromatographic methods suffer from coelutions of macromolecules with similar molar masses, limited sensitivity and time-consuming sample preparations. Thus, there is a quest for new methods and chromatographic columns to enable fast, selective and sensitive quantification of urinary myoglobin.[5,13]

Polymer monolithic columns have been used since the 1990s as either efficient stationary phases for the fast separation of proteins[14,15] or sorbents for solid phase extraction (SPE).[16-19] Porous polymer monoliths are easily synthesized using monomers, crosslinkers, porogenic solvents, and radical initiators.[20,21] Interconnected macropores measuring 1-2 μm define a surface area of only a few tens of $m^2\ g^{-1}$, facilitating the fast separation of proteins by the predominance of fast convective mass transport over slow diffusion.[22] The time and temperature of the polymerization reaction, the porogen solvent selection,

*e-mail: jcmasini@iq.usp.br
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and the monomer/porogen solvent ratio control the pore structure.[23]

Thermal free radical polymerization is the most common strategy to form monoliths inside supports such as stainless steel tubes, fused silica capillaries and polyetheretherketone (PEEK) tubes. Thermal polymerization takes several hours to achieve acceptable yields and reproducibility. In UV-transparent supports, such as polypropylene (PP), ethylene tetrafluoroethylene (ETFE), and fluorinated ethylene propylene (FEP), the polymerization can proceed in only a few tens of minutes by irradiating the capillary containing the polymerization mixture and the radical initiator.[7,24-26] The versatility of polymerization techniques enabled the construction of monoliths in various formats and dimensions, from the > 100 μm internal diameter (i.d.) capillaries to the conventional analytical 4.6 mm i.d. columns, thus including the microbore (0.5 to 1.0 mm i.d.) and narrow-bore (> 1.0 mm i.d.) columns.[23]

Former papers described the construction of monolithic columns of different sizes confined in plastic and Silcosteel® tubes using ethylene dimethacrylate (EDMA) or divinylbenzene (DVB) as crosslinker and butyl methacrylate (BMA), lauryl methacrylate (LMA), glycidyl methacrylate (GMA), or styrene (STY) as the functional monomer.[27,28] To our best knowledge, this is the first paper describing the one-step preparation of a highly nonpolar poly(SMA-*co*-EDMA) (SMA: stearyl methacrylate) column inside ETFE and Silcosteel® tubes. These columns worked to extract myoglobin from urine samples, followed by chromatographic analysis in the reversed-phase chromatographic mode. We demonstrated for the first time the use of narrow-bore (1.50 mm i.d.) poly(SMA-*co*-EDMA) in a low-cost and portable sequential injection chromatograph (SIC) and compared the results with those obtained in a conventional LC using a microbore (1.0 mm i.d.) poly(SMA-*co*-EDMA) column after concentrating myoglobin in the narrow bore column.

Experimental

Reagents

Stearyl methacrylate (SMA) and ethylene dimethacrylate (EDMA) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and purified by passing them through a basic aluminum oxide column to remove polymerization inhibitors. 3-(Trimethoxysilyl)propyl methacrylate (TMSPM), 1-propanol, 1,4-butanediol (porogenic solvents), benzophenone (BP), and 2,2-dimethoxy-2-phenyl acetophenone (DMPAP) and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich and used as received. Methanol (MeOH), acetonitrile (ACN), acetone, and ethyl alcohol were of high-performance liquid chromatography (HPLC) grade from J.T. Baker (Avantor Performance Materials, PA, USA). Lysozyme from chicken eggs, carbonic anhydrase from bovine pancreas, and myoglobin from horse skeleton muscle were from Sigma-Aldrich (St Louis, MO, USA). The protein stock solution (1.0 mg mL^{-1}) was prepared in 0.1 mol L^{-1} ammonium acetate (pH 7.0) and filtered through 0.45 μm cellulose acetate syringe filters before the chromatographic analyses. Deionized water (resistivity > 18 MΩ cm) from a Simplicity 185 system from Millipore (Billerica, MA, USA) was used to prepare all the solutions. Ethylene tetrafluoroethylene (ETFE) tubing with 0.75 and 1.5 mm i.d. from IDEX Health & Science housed the columns. Silcosteel® steel tubing with 1.59 mm outer diameter (o.d.) and 1.016 mm i.d. were purchased from Restek (Bellefonte, PA, USA).

Instrumentation

Chromatographic analyses were made in a Dionex Ultimate 3000 Dual Micro LC system (Dionex Softron GmbH, ThermoFisher Scientific, Germany) using dual micro DGP-3600 RS pumps with an SRD-3600 inline degasser, provided with a WPS-3000SL automatic sampler and a sampling loop for volumes between 0.1 and 20 μL. The chromatographic system was completed by a TCC 3000SD thermostated column compartment and an MWD-3000 UV-Vis detector coupled to a 2.5 μL semi-micro flow cell. Control of the instrument, data acquisition (20 Hz), and data processing was made with the software Chromeleon® 6.8. Connections of the column tube to the analyzers were made with P-702 PEEK unions, XP-335 PEEK nuts and P-300 ETFE ferrules from IDEX Health and Science (Oak Harbor, WA, USA). Photographing and photopolymerization were made in a Specrolinker XL-1000 UV-crosslinker from Spectronics Corporation (Westbury, New York, USA) provided with five 8-W, 254 nm lamps.

A SIChrom™ accelerated liquid chromatograph was provided by FIAlab® Instruments (Bellevue, WA, USA). The FIAlab 5.1 software synchronized the movements of the syringe pump, selection valve (Figure 1) and data acquisition (12 Hz) from the UV detector. Connections of the column tube to the chromatographic system were made with P-702 PEEK unions, XP335 PEEK nuts, and P-300 ETFE ferrules from IDEX Health and Science (Oak Harbor, WA, USA). Spectrophotometric measurements were carried out with a 1-cm-optical-path SMA-Z-10 μvol PEEK flow-through cell (6 μL of internal volume) from FIAlab Instruments. Detection was made at 400 nm using a 200-850 nm USB 4000 spectrophotometer (Ocean Optics,

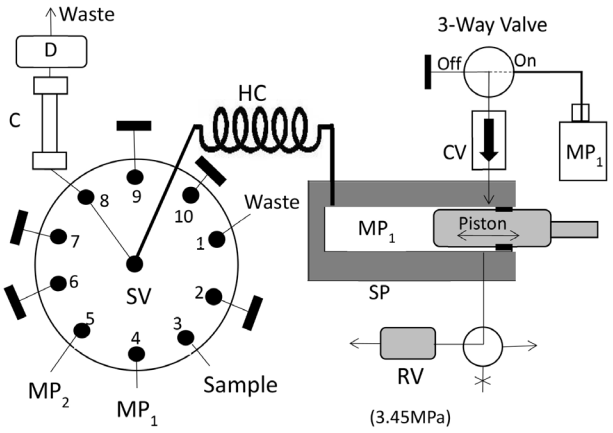

Figure 1. Sequential injection chromatograph for determination of myoglobin. SP = syringe pump, RV = 3.45 MPa relief valve, CV = check valve, HC = holding coil (4 m × 0.8 mm i.d.), SV = selection valve, C = 50 × 1.50 mm poly(SMA-*co*-EDMA) monolithic column, D = spectrophotometric detector (400 nm) connected to a 1-cm-pathlength 6-µL flow cell, MP1 = 5% ACN in 0.1% TFA, MP2 = 60% ACN in 0.1% TFA. All percentage refers to volumetric fractions.

Dunedin, FL, USA) with a grating of 600 lines mm^{-1}, resolution of 1.5 nm, and a slit width of 25 µm (height of 1000 µm). A DH 2000 deuterium tungsten halogen lamp (Mikropack GmbH, Germany) was used as the light source. Two 600-µm-diameter UV-Vis optical fibers (20 inches long) were used to transmit radiation from the source to the flow cell and spectrophotometer.

Scanning electron microscopy (SEM) was made with a Fesem Jeol JSM -740 1 F instrument (Jeol Ltda, Tokyo, Japan). Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) spectra of the inner wall of the ETFE tubes were obtained from 540 to 4000 cm^{-1} in a Frontier FT-IR instrument from PerkinElmer (Waltham, MA, USA).

Functionalization of the inner wall of the ETFE tubes

Briefly,150-mm long ETFE tubes were washed with ethanol and acetone and dried under N_2. A 20 wt.% BP solution in MeOH was sonicated (10 min) and purged with N_2 (10 min). This solution filled the tubes, which were closed in both ends and irradiated for 20 min under 254 nm at 120 mJ cm^{-2}. The closed tubes were positioned 2.5 cm apart from the UV lamps, and a rigid plastic plate covered with reflexive aluminum foil was placed just behind the columns. After this first step, the tubes were washed with methanol, dried with N_2, and filled with a 30 wt.% EDMA in methanol, previously sonicated (10 min), and purged with N_2 (10 min).[24] The tubes were closed and irradiated for 40 min (254 nm at 120 mJ cm^{-2}), keeping a distance of 2.5 cm from the lamps and the reflexive surface behind the columns. All the reactions were made with the photo-reactor in the vertical position. After polymerization, the column was cut to 10 cm long and flushed with MeOH to remove the unreacted monomers and the pore-forming solvents.

Functionalization of the inner wall of Silcosteel® tubes

The inner wall of the tubing was sequentially washed with ethanol, water, 0.2 mol L^{-1} NaOH (30 min at 0.25 mL min^{-1}), water, 0.2 mol L^{-1} HCl (30 min at 0.25 mL min^{-1}), water and ethanol. Next, the 20 wt.% TMSPM in 95% (v v^{-1}) ethanol (apparent pH adjusted to 5.0) derivatizing reagent, previously sonicated for 5 min, was pumped through the tube for 60 min at 0.25 µL min^{-1}.[28] The ends of the TMSPM-filled tube were closed with pieces Pharmed® peristaltic pump tubes sealed with solid PTFE tubes and heated overnight at 60 °C for 2-h inside the oven of a gas chromatograph. Finally, the tube was washed with acetone and dried under a flow of N_2.

Preparation of the monoliths

A polymerization mixture containing 24 wt.% SMA, 16 wt.% EDMA, 45.5 wt.% 1-propanol, and 14.5 wt.% 1,4 butanediol was prepared in a 2-mL amber vial in the presence of 1.0 wt.% initiator (relative to the monomers). Whereas the UV-mediated polymerization used DMPAP as an initiator, the thermal synthesis used azobisisobutyronitrile (AIBN). The mixture was sonicated for 10 min and purged with N_2 for another 10 min. The activated ETFE and Silcosteel® tubes were filled with the polymerization mixtures and closed at both ends. The ETFE tube was irradiated for 20 min under 254 nm at 120 mJ cm^{-2}. The Silcosteel® tubes were heated at 50 °C for six hours inside the oven of a gas chromatograph. The column ends were cut to provide 100 mm long columns and flushed with ACN at 500 µL min^{-1} until a constant pressure, thus removing the unreacted monomers and porogenic solvents. The columns were stored in ACN until use.

Samples

Urine samples were voluntarily provided before and 10 min after one hour of anaerobic strength training, and a previous consent has been obtained from all subjects. The samples were buffered with 0.1 mol L^{-1} ammonium acetate (pH 7.0) at a 9:1 volumetric ratio and filtered through 0.22 µm syringe filters.[5] The samples were then analyzed before and after spiking with known concentrations of myoglobin by reversed-phase liquid chromatography (RPLC) in both SIC and microbore LC. Just before the

SPE experiments, urine samples were conditioned in 0.1% (v v^{-1}) TFA.

The sample preparation for the offline SPE consisted of loading 2.0 mL of the filtered and 0.1% TFA conditioned samples in the 50 ×1.50 mm i.d. ETFE columns, washing with 5% ACN in 0.1% TFA and then back-elution with 0.50 mL of 60% ACN in 0.1% TFA. A sample aliquot of 5 μL was analyzed by the microbore HPLC.

Sequential injection chromatography

The sequential injection chromatography instrument is illustrated in Figure 1, and details on its functioning are given elsewhere.[29] After filling/conditioning the syringe pump, holding coil, column, and flow cell with the mobile phase composed of 5% (v v^{-1}) ACN in 0.1% (v v^{-1}) TFA, labeled as MP1, the sampling line was filled with the sample solution, discarding the sample excess entering the holding coil (this step was made manually, although it could be automated via software). Elution mobile phase was composed of 60% (v v^{-1}) ACN in 0.1% (v v^{-1}) TFA, labeled as MP2.

Analysis of urine samples followed a 5-step procedure (Table 1) which consisted of aspirating 1500 μL of MP1 inside the syringe pump (step 1), followed by sequential aspiration of 500 μL of MP2 (step 2), 500 μL of MP1 (step 3), and 1000 μL of sample/standard solution (step 4). Finally, the syringe pump emptied the syringe by pumping the sample, and the interpenetrated MP1, MP2 and MP1 zones through the monolithic column and detection flow cell at 10 μL s^{-1} (steps 5-7), monitoring the absorbance at 400 nm. Retention times, peak areas and heights were computed using the signal processing feature of the Origin 8.5.1 software.[30]

HPLC analysis

The poly(SMA-*co*-EDMA) column (100 mm × 1.0 mm i.d.) was used for chromatographic separations based on the use of two mobile phases: mobile phase A: 0.1% (v v^{-1}) TFA in water and mobile phase B: 0.1% (v v^{-1}) TFA in ACN. The elution program was 0 to 1 min: isocratic elution with 5% B; 1 to 7 min: linear gradient from 5 to 50% B; 7.0 to 7.1 min: linear gradient from 50 to 5% B; 7.1 to 10 min: isocratic elution with 5% B.

The injection volume was 5 or 10 μL, the flow rate was 1.0 mL min^{-1}, the detection wavelength was 205 nm and the column temperature was 60 °C. The standard stock solutions were prepared in 10 mM ammonium acetate (pH 7.0). All the solutions were filtrated through a 0.22 μm syringe filter and stored at 4 °C before analysis.

Results and Discussion

Poly(SMA-*co*-EDMA) characterization

Scanning electron microscopy

The challenge of using ETFE or other fluorinated plastic housing is their hydrophobicity and low reactivity, which makes it difficult to anchor the stationary phase to the tubing inner wall without leaving empty spaces that deteriorate the separation performance.[26] To achieve a covalent binding of the monolith to the inner wall of ETFE tubing, we used the two-step vinylization procedure, first proposed by Stachowiak *et al*.[31] to anchor monoliths into polypropylene, further adapted by Catalá-Icardo *et al*.[32] to ETFE. Shortly, in the first step, benzophenone is photografted on the polymer to create a surface photoinitiator. This first modification does not change the physical appearance of the ETFE tube. In the second step, the initiator mediates the photografting of a thin layer of cross-linked poly(EDMA), thus forming an assortment of terminal vinyl groups to anchor the monolith inside the housing. After this second modification and tube washing with methanol, a dense white layer of poly(EDMA) bonded to the inner surface is naked-eye observed.

SEM image of a radial section of the 1.5 mm i.d. ETFE tubing shows a homogeneous surface of the UV-polymerized poly(SMA-*co*-EDMA) monolith (Figure 2a). Figure 2b emphasizes the functionalized interface between the ETFE inner wall and the monolith. The unreacted poly(EDMA) double bonds copolymerize with SMA and EDMA in

Table 1. SIC procedure for determination of myoglobin in urine samples

Step	SV port	SP command	Comment
1	9	aspirate 1500 μL at 50 μL s^{-1}	filling the syringe pump with MP1
2	5	aspirate 500 μL at 50 μL s^{-1}	aspiration MP2 into HC for analyte elution
3	4	aspirate 500 μL at 50 μL s^{-1}	aspirations of MP1 into HC to SPE washing
4	3	aspirate 1000 μL at 50 μL s^{-1}	sample aspiration
5	8	empty at 10 μL s^{-1}	injecting sample, washing (MP1), eluting (MP2) and reconditioning (MP1) solutions through the column and detector

SV: selection valve; SP: syringe pump; HC: holding coil; MP1: mobile phase 1 (5% acetonitrile in 0.1% trifluoroacetic acid), MP2: mobile phase 1 (60% acetonitrile in 0.1% trifluoroacetic acid).

the presence of porogenic solvents, thus anchoring the poly(SMA-*co*-EDMA) monolith. The core of the monolith prepared in the 0.75 and 1.50 mm i.d. ETFE tubes do not show any significant difference, suggesting the 20 min polymerizations at 254 nm and 120 mJ cm^{-2} radiation was enough to produce a uniform structure of 2-3 μm microglobules intercalated by interconnected 5 to 10 μm flow-through pores.

Figure 2e shows the interface of the Silcosteel® tube anchoring the poly(SMA-*co*-EDMA) monolith, whereas Figure 2f shows the core of monolith obtained by 24-h thermal polymerization. The SEM images suggest that the thermally produced monolith keeps the large 5 to 10 μm flow-through pores but with globules larger than those obtained by photopolymerization.

ATR-FTIR

The ATR-FTIR spectra of the poly(SMA-*co*-EDMA) monoliths show the typical bands at 2850 and 2925 cm^{-1} due to C–H stretching and CH_3 symmetric stretching, respectively (Figure 3). The bands at 1465 and 1390 cm^{-1} correspond to CH_2 and CH_3 bending in the C_{18} chain. The 1725 cm^{-1} band can be assigned to stretching C=O, whereas the 1145 cm^{-1} band comes from stretching C–O bonds in the methacrylate structures. The weak band at 1640 cm^{-1} due to C=C stretching confirms the polymerization reaction through the vinyl functionalities, as confirmed by comparing the ATR-FTIR of the poly(SMA-*co*-EDMA) with the IR spectra of the monomers shown in the Supplementary Information (SI) section (Figure S1).

Permeability

The permeability of each column was computed according to the Darcy Law:

$$K_0 = \frac{L\eta\mu_s}{\Delta_P} \quad (1)$$

where K_0 is the permeability in m^2, L is the length of the column in m, η is the viscosity of the mobile phase (acetonitrile) in cp (1×10^{-3} N m^{-2} s^{-1}), μ_s is the linear

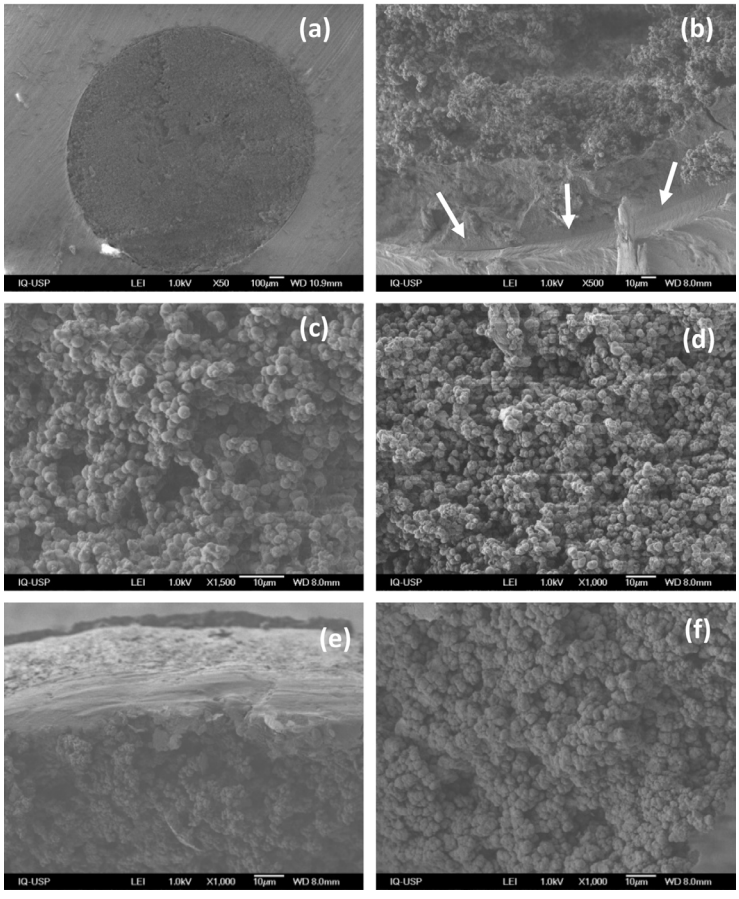

Figure 2. SEM images of a cross-section of the poly(SMA-*co*-EDMA) monoliths showing (a) the monolith housed in 1.5 mm i.d. ETFE tube; (b) interface between the ETFE tube and the monolith anchored in a layer of poly(EDMA) pointed by the arrows; (c) core of the monolith prepared in a 0.75 mm i.d. ETFE tube; (d) core of the monolith prepared in a 1.5 mm i.d. ETFE tube; (e) interface between the vinylized Silcosteel® tube and the monolith, and (f) the core of the monolith prepared inside Silcosteel® tube by thermal polymerization.

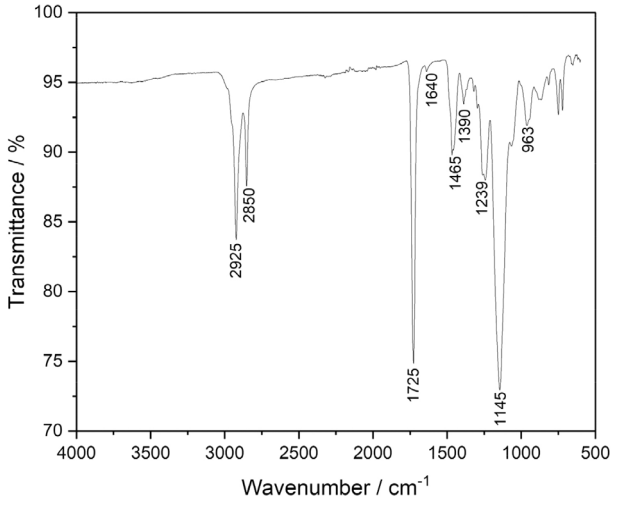

Figure 3. ATR-FTIR of the core of the poly(SMA-*co*-EDMA) monolith.

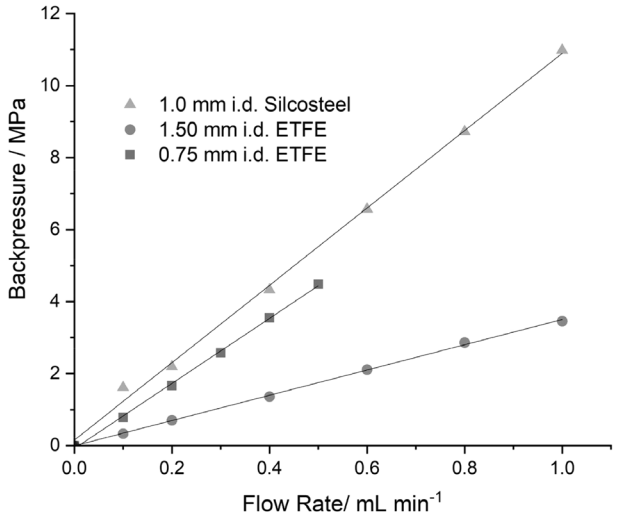

Figure 4. Back pressure as a function of the flow rate of 5% ACN in 0.1% TFA at 30 °C.

flow velocity (m s^{-1}) and Δ_p is the back pressure (N m^{-2}) (measured at a flow rate of 500 µL min^{-1}).

The permeability was (4.4 ± 0.1) × 10^{-14}, (6.75 ± 0.4) × 10^{-13} and (1.68 ± 0.07) × 10^{-13} m^2 for the columns with 1.5, 1.0, and 0.75 mm i.d., respectively. These permeabilities resulted from the large flow-through pores in the structure (Figure 2), enabling high flow rates even with micro and narrow bore columns. The relative standard deviation (RSD) of the permeabilities (three columns of each dimension) was 2.2, 5.9 and 4.1% for the 1.5, 1.0 and 0.75 mm i.d. columns, respectively, showing that both thermal and photopolymerization reactions produce columns with reproducible permeabilities.

The back pressure increased linearly with the flow rate (0.1% (v v^{-1}) TFA in ACN), at least up to 1.0 mL min^{-1}, reaching 3.45 and 10.98 MPa at 30 °C in the 1.5 mm i.d. ETFE and 1.0 mm i.d. Silcosteel® columns, respectively (Figure 4). The 0.75 mm i.d. column in ETFE was less robust than the others, especially regarding the connections with the chromatograph, which holds the column only up to a back pressure of 4.5 MPa (0.50 mL min^{-1}). Besides, they released small pieces of polymer that could damage the instrument and detector flow cell, so they were not studied further.

Separation of proteins by UV-RPLC

The large flow through pores and the absence of mesopores filled with stagnant mobile phases make the convection prevail over the slow diffusive transport in the polymer monoliths, thus enabling the efficient separation of slow-diffusion macromolecules such as proteins. This feature of the synthesized monolithic columns was demonstrated by separating lysozyme, myoglobin, and carbonic anhydrase. The column prepared in the 1.0 mm i.d. Silcosteel® tube exhibited the best performance, providing baseline separation of the three proteins (Figure 5). Thus, the application of the monoliths for urine analyses used the 1.5 mm i.d. ETFE column for SPE (since they provide a large sample capacity) and the Silcosteel® column as the analytical column.

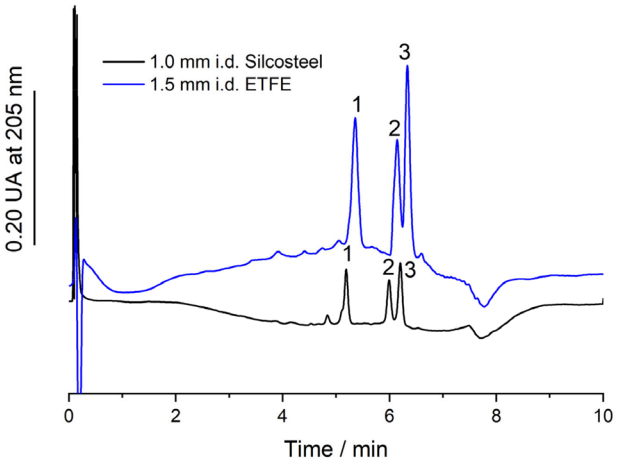

Figure 5. Blank-subtracted chromatograms of a mixture of (1) lysozyme, (2) myoglobin and (3) carbonic anhydrase (all at 75 µg mL^{-1}) under conditions described in "HPLC analysis" sub-section. The injected sample volumes were 5.0 and 20.0 µL in the 1.0, and 1.5 mm i.d. columns, respectively. Flow rate was 1.0 mL min^{-1} for the 1.0 mm i.d. Silcosteel® and 1.5 mm i.d. ETFE. The temperature was 60 °C.

The reproducibility of the columns was evaluated by comparing the peak areas and retention factor (k) for myoglobin obtained from duplicates of columns prepared by thermal polymerization in Silcosteel® tubes and a triplicate of the columns prepared by photopolymerization in the ETFE tubes (Table 2). The RSD of peak areas was < 7.25% considering triplicates of injections in

each column, whereas the RSD of retention factors was 15.1 and 10.9% for the ETFE and Silcosteel columns, respectively. Despite the high RSD, all the prepared columns separated the protein mix. From the statistical point of view, improvements in RSD would be achieved by the construction of a larger number of columns. Longer polymerization times would also improve the RSD because the reactions could approach the 100% conversion of monomers.

Table 2. Reproducibility of peak areas and retention factors (k) obtained for a 25 μg mL^{-1} myoglobin solution analyzed in different ETFE and Silcosteel® columns under the RPLC gradient (n = 3) described in "HPLC analysis" sub-section. The injected volumes in ETFE and Silcosteel columns were 5 and 2 μL, respectively

Column	Peak area	k
ETFE 1	3.14 ± 0.08	23.2 ± 0.6
ETFE 2	3.54 ± 0.12	30 ± 1
ETFE 3	3.12 ± 0.08	31 ± 1
RSD - ETFE / %	7.25	15.1
Silcosteel® 1	1.13 ± 0.03	31.1 ± 0.9
Silcosteel® 2	1.22 ± 0.07	26.7 ± 0.8
RSD - Silcosteel® / %	5.41	10.9

ETFE: ehylene tetrafluoroethylene; RSD: relative standard deviation.

Sequential injection determination of myoglobin

The SIC instrumentation was not able to separate the three proteins in the reversed-phase mode using the poly(SMA-*co*-EDMA) column due to the difficulty in creating a linear ACN gradient. However, this potential interference can be circumvented by detection at 400 nm since, at this wavelength, only myoglobin absorbs (Figure S2, SI section). The SIC methodology is indeed an automated SPE protocol to concentrate myoglobin, eliminate salts and other potential interferences and finally elute the protein to the detector flow cell. A 50 × 1.5 mm i.d. poly(SMA-*co*-EDMA) column in the ETFE tube was used, thus enabling the use of a flow rate of 600 μL min^{-1} during the washing/elution steps without overpressure issues, which are controlled in the SIC instrument by the relief valve that opens at pressures > 3.45 MPa (Figure 1).

Large sample volumes injected in high polarity solvents have been used in reversed-phase SIC methods to drive weakly polar analytes towards the apolar stationary phase, thus providing inline enrichment before separation.[33,34] In the present paper, peak areas increased with the sample volume up to 1000 μL (5.0 μg mL^{-1}), so this was the volume adopted in the SIC method. The SIC procedure created a steep gradient from 5 to 60% ACN in the presence of the ion pairing TFA before aspirating the sample volume inside the holding coil (steps 2, 3 and 4 as described in Table 1). Upon reverting the flow towards the column conditioned with 5% ACN in 0.1% TFA, hydrophobic interactions retain myoglobin in the poly(SMA-*co*-EDMA) column. The MP1 zone aspirated between the sample and MP2 zone elutes the sample matrix before the elution of myoglobin by MP2. These volumes of MP1 and MP2 (500 μL) are large enough, so their dispersion coefficients at the central zones inside the 0.8 mm i.d. holding coil approach the unity and provide a steep gradient as the interspersed zones reach the column and then the detector.[35]

Since the SIC configuration has only one selection valve (no injection valve), automated analyses were made using a unidirectional flow approach (Table 1). This procedure takes 400 s, implying a sampling throughput of 9 analyses *per* hour, including the inline sample enrichment, washing, elution and reconditioning. Acquisition data started only after the column loading and washing, so the chromatogram starts at 170 s (Figure 6). The mean retention time (Figure 6) was 273 ± 2 s, without discounting the initial SPE steps. The 0.8% relative standard deviation (RSD) in the retention time demonstrated the high precision of the pumping system and the concentration gradient formed in the holding coil. The RSD (n = 5) for the peak areas at 5.0, 10, and 50 μg mL^{-1} concentration levels were 7.1, 5.2 and 3.0%, respectively, confirming the good precision of the measurements.

A typical calibration curve was prepared using 5.0 to 60 μg mL^{-1} myoglobin solutions (Figure 6).

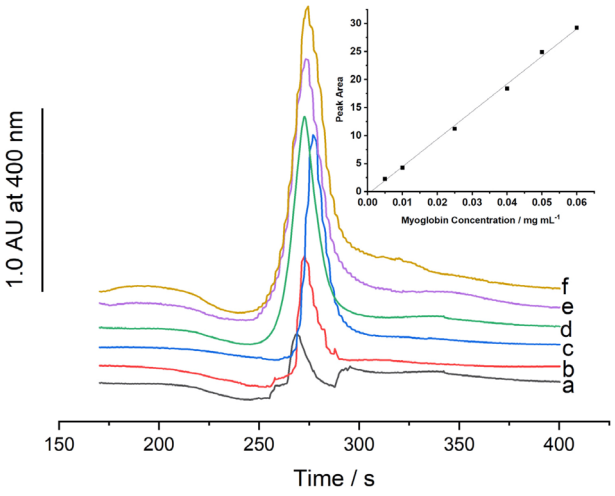

Figure 6. Chromatograms for the construction of a calibration curve (inset) for myoglobin concentrations of (a) 5.0, (b) 10, (c) 25, (d) 40, (e) 50 and (f) 60 μg mL^{-1}. The measurements were made by loading the column with 1.0 mL of standard solution, followed by washing and elution with a gradient of 5 to 60% ACN in 0.1% TFA prepared inside the holding coil of the SIC instrument (according to Table 1).

The peak areas increased linearly with the myoglobin concentration following the equation Peak area = (491 ± 11)C_{myo} – (0.5 ± 0.4), with correlation coefficient (R^2) = 0.997, $S_{y/x}$ (standard deviation of *y*-residuals = 1.96), denoting an acceptable linearity. Peak heights increased linearly with concentration up to 50 μg mL^{-1} resulting in the calibration curve Peak area = (48 ± 5)C_{myo} + (0.08 ± 0.07) with R^2 = 0.98 and $S_{y/x}$ = 0.018. The limits of detection (LOD) and quantification (LOQ) values were computed as $LOD = 3S_{y/x}/m$ and $LOQ = 10S_{y/x}/m$,[36] where m is the slope of the calibration curve using peak height measurements for the 5.0, 10, and 25 μg mL^{-1} concentrations, resulting in 1.1 and 3.7 μg mL^{-1}, respectively. This LOD is suitable for the measurement of concentration levels reported in cases of crush trauma (32.9 μg mL^{-1}),[37] military training (6.4 to 410 μg mL^{-1}),[38] runners following marathons (52 μg mL^{-1}),[39] rugby competitors (100 μg mL^{-1})[1] and myocardial infarction (3.0 mg mL^{-1}).[9]

Myoglobin in urine samples by SIC

SIC chromatograms of urine samples had no detectable myoglobin concentrations before and after training. The chromatograms of the blank, sample and spiked sample (Figure S3, SI section) show similar blank and urine chromatograms, denoting that the SIC procedure and the choice of 400 nm as the detection wavelength provide a clean baseline free of interferences. The accuracy of measurements was made by enriching the samples with known amounts of myoglobin and analyzing the samples with quantification by the external calibration curves. The recoveries for enrichment levels of 10, 25 and 50 μg mL^{-1} were 103.4, 104.47, and 98.75%, respectively.

HPLC analysis

Before proceeding with the SPE experiments, a calibration curve was constructed for myoglobin concentrations between 2.5 and 50 μg mL^{-1} using the 1.0 mm i.d. Silcosteel® column and injecting 5 μL of standard solutions. The peak areas increased linearly with the myoglobin concentrations according to the equation Peak area = (49 ± 2)C_{myo} – (0.02 ± 0.04) with R^2 = 0.997, and $S_{y/x}$ = 0.024, thus implying LOD and LOQ of 1.5 and 4.8 μg mL^{-1}, respectively. Since these values are of similar magnitude to those obtained by SIC, but using a sample volume 200-fold lower, it was expected a significant increase in detectability by implementing the SPE step.

Different from SIC, the HPLC method used an offline SPE strategy. Known volumes of 5.0 μg mL^{-1} myoglobin (1.0, 2.0, 3.0, and 4.0 mL) were pumped through the column (50 × 1.5 mm i.d. ETFE tube) at 0.25 mL min^{-1}, followed by washing with 1.0 mL of 5% ACN in 0.1% TFA. Myoglobin was back-eluted (0.50 mL of 60% ACN in 0.1% TFA) to a vial and analyzed (5 μL) by HPLC using the 100 × 1.0 mm i.d. Silcosteel® column. Recoveries were estimated from the peak area measurements and the theoretical enrichment factors (EF) 2, 4, 6, and 8 would be expected for the sample volumes of 1.0, 2.0, 3.0, and 4.0 mL, respectively (Figure S4, SI section). For these volumes, the experimental EF factors were 2.1, 4.0, 5.6, and 6.0, indicating that breakthrough occurs for sample volumes > 2.0 mL. Thus, for the HPLC analyses, loading a sample volume of 2.0 mL followed by washing and elution with 60% ACN in 0.1% TFA was adopted for the next steps.

The effect of elution volume on the recoveries was investigated after loading the SPE column with 1.0 mL of a 50 μg mL^{-1} solution. Back elution with 0.35, 0.40, 0.45, and 0.50 mL of 60% (v v^{-1}) ACN in 0.1% (v v^{-1}) TFA, at 35 μL min^{-1}, revealed that the recovery reached 100% only with 0.50 mL (Figure S4) so that this volume was used for the construction of calibration curves including the SPE and the sample analyses. Under these conditions, a calibration curve for myoglobin concentrations between 1.0 and 7.5 μg mL^{-1} fitted the linear equation Peak area = (111 ± 7)C_{myo} – (0.01 ± 0.03) with R^2 = 0.989 and $S_{y/x}$ = 0.0048, resulting LOD = 0.13 μg mL^{-1} and LOQ = 0.43 μg mL^{-1}. The ratio of the slope of the calibration curves with SPE and without SPE implied an EF of 2.3 and 10-fold enhancement in detectability. This limit of detection is about 2-fold lower than that reported by Lindsay *et al.*[5] and about 4-fold lower than that reported by Al-Rimawi[40] both using RP-HPLC methods.

Analysis of urine samples

No myoglobin concentration was detected in the samples collected before the training. The concentrations found after training appear in Table 3. The urine sample from Individual 1 had a myoglobin concentration of 6.0 ± 0.4 μg L^{-1}, a concentration close to the LOQ of the HPLC method. After the SPE protocol, an undistinguishable concentration value was found, showing the accuracy of the method (Figure 7a). The sample from Individual 2 had quantifiable concentrations without the need for SPE. Individual 3 provided a sample with detectable but not quantifiable myoglobin concentration and the SPE protocol made it possible to bring the extracted solution to the linear response range (Figure 7b).

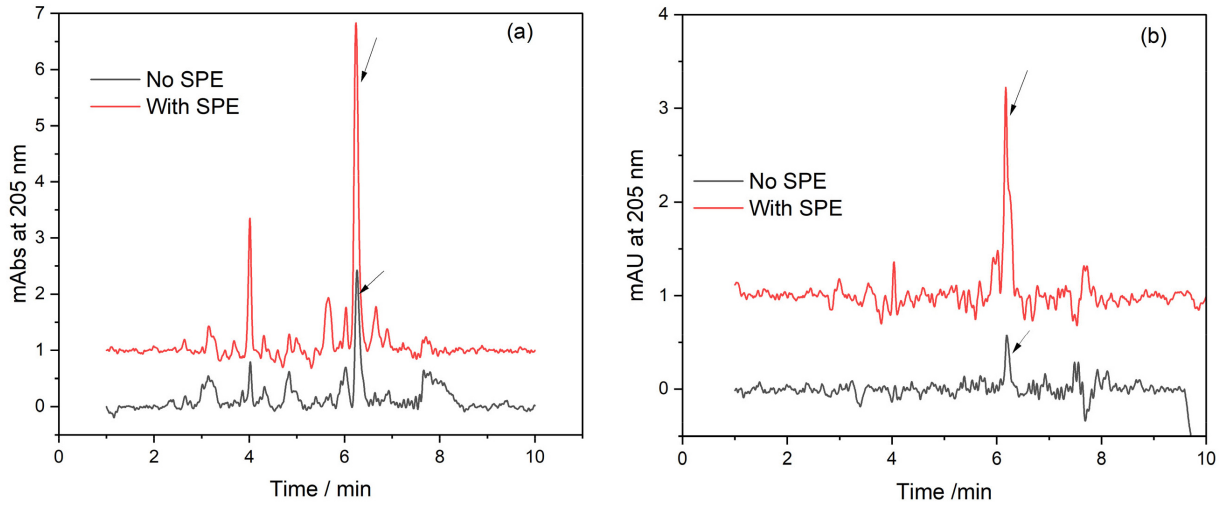

Figure 7. Blank subtracted chromatograms of urine samples of (a) individual 1 and (b) individual 3 before and after SPE with the poly(SMA-*co*-EDMA) columns. The arrows show the myoglobin peak. Chromatographic conditions were described in "HPLC analysis" sub-section; sample volume = 5 µL, temperature = 60 °C.

Table 3. Urinary myoglobin concentrations in three samples before and after SPE (n = 3)

Sample	Without SPE / (µg L^{-1})	With SPE / (µg L^{-1})
1	6.0 ± 0.4	6.0 ± 0.5
2	28 ± 1	not required
3	< LOQ	2.3 ± 0.4

SPE: solid-phase extraction; LOQ: limit of quantification.

The accuracy of the HPLC method was investigated by spiking the samples with 10, 25, and 50 µg mL^{-1}, resulting in recoveries of 105, 98 and 99%, respectively with an RSD < 12% for the 10 µg mL^{-1} level.

Conclusions

Simple and easy preparation of polymer monolithic columns in different dimensions and molds enabled the development of reversed-phase chromatographic methods to determine urinary myoglobin, using SPE as required. Both SIC and HPLC methods could benefit from the monolithic columns. Although SIC was not able to provide reversed-phase separation of lysozyme, myoglobin and carbonic anhydrase, it automates all the SPE steps of column conditioning, sample loading, washing and elution/detection. For this specific application, the selectivity was achieved by detection at 400 nm, a wavelength selective to myoglobin. The back pressure limitations of SIC demanded the use of a narrow-bore 50 × 1.50 mm i.d. column constructed in an ETFE tube by photopolymerization. The HPLC analysis benefited from the efficient separation of proteins in a gradient of ACN in 0.1% TFA provided by the microbore poly(SMA-*co*-EDMA) column constructed in Silcosteel® tube. Combining SPE and HPLC analysis provided an EF 2.3, with a 10-fold improvement in detectability, reaching LOD and LOQ of 1.3 and 4.3 µg L^{-1}, respectively, which are low enough to detect harmful increases of urinary myoglobin, thus having potential application in medical diagnosis. An automated online SPE RPLC method would be easily implemented with the monolithic columns provided a rotary automated valve is available. While the conventional RPLC method provides fine chromatographic separation and versatility to choose the column dimensions, the SIC method shines by its portability, low instrumentation cost and reduced solvent consumption.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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