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Latex-functionalized monolithic columns for the separation of carbohydrates by micro anion-exchange chromatography

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Abstract

A novel stationary phase for micro ion chromatography has been prepared by coating a porous poly(butyl methacrylate–*co*-ethylene dimethacrylate–*co*-2-acrylamido-2-methyl-1-propanesulfonic acid) monolith with quaternary amine-functionalized latex particles via simple electrostatic binding. This stationary phase enabled the separation of saccharides in the mobile phase with a high-pH value consisting of aqueous ammonia solution in anion-exchange mode using evaporative light scattering for detection. Effects of both porous properties of the monolithic stationary phase and chromatographic conditions on the separation ability were studied. Under optimized conditions, an efficient separation of seven saccharides was achieved in less than 10 min. The stationary phase also enables the separation of saccharides obtained by the enzymatic hydrolysis of corn starch.

Keywords: Monolithic columns; Stationary phases, LC; Carbohydrates; Latex

1. Introduction

Recent progress in life science has revealed the importance of many types of oligo- and polysaccharides in animal bodies and has given rise to much interest in the biological functions of these special types of glycans [1,2]. For example, glycosylation, the covalent attachment of saccharides to specific amino acid residues, is one of the most common post-translational modifications of proteins. Also, recent glycoproteinaceous drugs produced by biotechnology pose a potential problem in that they may have a different glycosylation profile compared to their native counterparts leading to change in specificity in recognition of receptors. Furthermore, advances in immunology and pathology require insight into fine structure of various polysaccharides in bacteria and plants. With these trends in glycobiology and glycotechnology, considerable demand can be traced for the development of methods for carbohydrate analysis, in particular those, which are amenable to miniaturization. These methods must enable high-resolution separations because most samples comprise a number of structurally similar isomers or homologues. Currently, anion-exchange chromatography (AEC) at high-pH, which benefits from weak acidic nature of carbohydrates, is the method of choice for the analysis [3]. Since these compounds do not possess a suitable chromophore, pulsed amperometric detection has been introduced as a highly sensitive and selective detection method [3–5]. These developments led to the wide acceptance of the state-of-the-art technique for carbohydrate analysis called high performance or high-pH anion-exchange chromatography with pulsed amperometric detection.

The most common ion-exchange resins used in chromatographic separations of carbohydrates are agglomerated resins consisting of a internal core bead to which a monolayer of microparticles containing suitable functional groups are attached via electrostatic binding [6]. For example surface-sulfonated non-porous poly(styrene–divinylbenzene) beads attract and bind a layer of latex particles bearing quaternary ammonium functionalities. The resulting stationary phase then behaves as an anion-exchanger. Since the mono-

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layer of functionalized latex particles is thin, excellent chromatographic performance is achieved due to the very short diffusion path lengths. Despite the remarkable success of these agglomerated resins in traditional ion chromatography, their use in micro and nanoscale liquid chromatography is challenging, mostly because of the practical difficulties associated with uniform packing and retaining of these particles in columns or channels of small dimensions [7,8].

In contrast to packed columns, porous polymer monoliths can be formed directly within a specific location inside a narrow bore capillary column or microfluidic channel [9]. Therefore, they are rapidly gaining acceptance as stationary phases, particularly for miniaturized systems [10–12]. In recent years, our group has been extensively involved in the development of these materials and demonstrated several approaches to control their surface chemistry. These include copolymerization of suitable functional monomers [13], polymer analogous reactions of reactive functionalities of incorporated monomer units [14], and functionalization via thermal [15] or photoinitiated [16] grafting. In this report, we are presenting another approach to modification of the surface chemistry of monolithic materials using electrostatic attachment of functionalized latex particles. Based on the success of similar particulate materials used in conventional ion chromatography, this work presents the application of latex-coated monolithic capillary columns to the separation of saccharides.

2. Experimental

2.1. Materials

2-Acrylamido-2-methyl-1-propanesulfonic acid (99%, AMPS), 1,4-butanediol (99%), 1-propanol (99%), 2,2′-azobis(2-methylpropionitrile) (99%, AIBN), ammonium hydroxide, methanol, iodomethane, and 3-(trimethoxysilyl)propyl methacrylate (98%) were purchased from Aldrich (Milwaukee, WI, USA). Basic alumina (Brockman activity I, 60–325 mesh) was obtained from Fisher Scientific (Pittsburgh, PA, USA). D(+)Fructose, D(+)galactose, D(+)glucose, maltose, D(+)mannose, sucrose and D(+)xylose, were purchased from Sigma (St. Louis, MO, USA). Butyl methacrylate (99%, BuMA), ethylene dimethacrylate (98%, EDMA) (both Aldrich) were purified by passing them through a bed of basic alumina (Brockman activity I, 60–325 mesh) to remove inhibitors and distilled under reduced pressure. All other reagents were of the highest available grade and used as received.

A “real life” sample of saccharides was obtained by hydrolysis of 0.1 g corn starch dissolved in 1 mL of human saliva and diluted to 10 mL with ultrapure water. The mixture was thermostatted at 37 °C under gentle agitation for 12 h, then centrifuged, and the supernatant immediately injected in the column.

2.2. Preparation of porous polymer monoliths in fused silica capillaries

Polyimide coated fused silica capillaries (250 μm i.d., Polymicro Technologies, Phoenix, AZ, USA) were rinsed with acetone and water using a syringe pump, activated with 0.2 mol/L sodium hydroxide for 30 min, washed with water, then with 0.2 mol/L HCl for 30 min, then with water again, and finally with ethanol. A 20% (w/w) solution of 3-(trimethoxysilyl)propyl methacrylate in 95% ethanol adjusted to pH 5 using acetic acid was pumped through the capillaries at a flow velocity of 1 mm/s for 1 h, the capillary was washed with ethanol, dried in a stream of nitrogen, and left at room temperature for 24 h. Using a syringe, the 25 cm long surface modified capillary was completely filled with a polymerization mixture consisting of BuMA (22%), EDMA (16%), AMPS (2%), and AIBN (1% with respect to monomers) dissolved in a mixture of 1,4-butanediol with 1-propanol (total of both 50%), and water (10%) serving as the porogenic solvent. The ends were sealed and the capillary heated in a water bath at 70 °C for 24 h. The monolith in the capillary was then washed with methanol pumped through at a flow rate of 1 μL/min for 12 h, followed by washing with water for 2 h at the same flow velocity.

2.3. Functionalization of the monoliths with latex beads

Latex particles (60 nm diameter) with tertiary amine functionality were obtained from Bangs Labs. (Fishers, IN, USA). The amine groups were quaternized via reaction with iodomethane according to a procedure described previously [17,18]. Briefly, 0.2 g of particles were suspended in 1.5 mL of dimethylformamide to which had been added 50 μL of 1,2,2,6,6-pentamethylpiperidine. Following short sonication, 0.15 mL of methyl iodide was added. This suspension was heated at 60 °C with stirring overnight. The modified particles were isolated by filtration through a 0.03 μm polycarbonate membrane (Whatman), washed successively with water, methanol, triethylamine and methanol, and then dried in a vacuum oven at room temperature. An aqueous suspension of these particles (∼0.05 g/mL) was prepared by sonication of the solid in water and pumped through the monolith at a flow rate of 1 μL/min for 2 h. The monolith was then rinsed with water and conditioned with the eluent (16–320 mmol/L NH_4OH) for 1 h at a flow rate of 5 μL/min. Two 10 cm long capillary columns were then cut from the precursor monolith.

2.4. Porosity measurements

Simultaneously with the polymerization in capillary, the same mixture was also polymerized in a glass vial to obtain sufficient amount of monolithic polymer for the determination of the pore volume and pore size distribution using an Autopore III 9400 mercury intrusion porosimeter (Micromeritics, Norcross, GA, USA). Another monolith was prepared in a larger diameter glass tube (2 mm i.d.), washed,

coated with the latex particles, and used again for determination of surface area. The surface area was calculated from the BET isotherms of nitrogen adsorption and determined using an ASAP 2010 analyser (Micromeritics).

2.5. Chromatography

A Waters 515 pump (Milford, MA, USA) was connected to a M472 variable flow splitter and a M485 microinjection valve with a 100 nL sample loop (both Upchurch Scientific, Oak Arbour, WA, USA). The column effluent was passed directly into a model PL ELS 1000 evaporative light scattering detector (Polymer Labs., Amherst, MA, USA). The system was controlled and data acquired by Millenium 3.2 software (Waters).

3. Results and discussion

The preparation of monolithic capillary columns used in this study is a simple straightforward process. The polymerization mixture containing monomers—butyl methacrylate, ethylene dimethacrylate, and 2-acrylamido-2-methyl-1-propanesulfonic acid, an initiator 2,2'-azobis(2-methylpropionitrile), and a mixture of 1,4-butanediol, 1-propanol, and water as the porogenic solvent is filled in capillary, which surface was "vinylized" using a silane reagent, and the polymerization is triggered by heating to 70 °C for 24 h. Fig. 1 shows the cross-section of the monolithic capillary column and clearly demonstrates that the monolith fills completely the available volume. This also prevents the undesirable flow around the monolith in the free space next to the capillary wall that would be observed if the polymer were not attached to the wall [19].

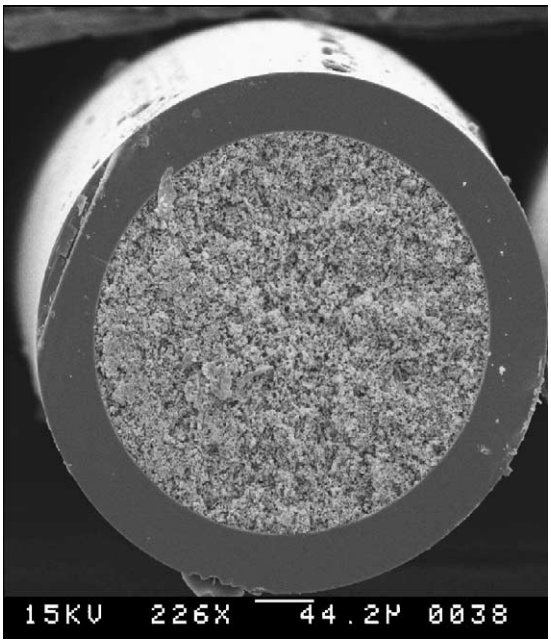

Fig. 1. SEM image of cross-section in 250 μm i.d. capillary column containing BuMA–*co*-EDMA–*co*-AMPS monolith with a median pore size 2.30 μm.

3.1. AMPS content and porous properties

Obviously, it would be desirable to incorporate as much AMPS monomer in the polymer monolith as possible and to maximize the amount of sulfonic acid functionalities on the surface of the pores that in turn would increase its coverage with the latex beads. However, in our early studies with similar monoliths designed for capillary electrochromatography we found that the pores are blocked at contents of AMPS in the polymer exceeding 5% due to excessive swelling [20]. In order to avoid this problem and to limit the number of system variables, all monoliths prepared in this preliminary study contained 5% AMPS.

Pore size is the variable that controls the resistance to flow through the monolith. Fig. 2 shows that varying proportions of 1,4-butanediol and 1-propanol (porogenic solvents) in the polymerization mixtures enables the preparation of monoliths with pore sizes in a range of 162–4200 nm. However, rather high column back pressures were observed for monolithic columns with the median pore diameter less than 1 μm. In addition, these columns tend to get blocked when attempting to apply the latex particles dispersion. Hence, only those monoliths with average pore diameter in the range of 1–4 μm were tested in this study. Their pore size distribution curves are shown in Fig. 3.

3.2. Functionalization with latex particles

We assumed that successful functionalization of pore surface within the monolithic columns with the latex particles

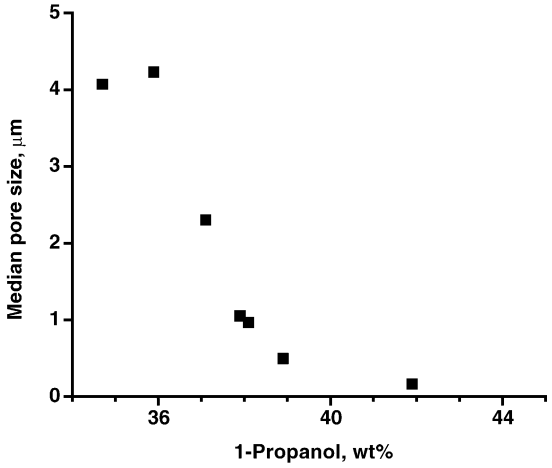

Fig. 2. Effect of percentage of 1-propanol in polymerization mixture on median pore size. Polymerization mixture: butyl methacrylate 22%, ethylene dimethacrylate 16%, 2-acrylamido-2-methyl-1-propanesulfonic acid 2%, AIBN (1% with respect to monomers) 1,4-butanediol and 1-propanol (total of both 50%), and water (10%), polymerization time 24 h, temperature 70 °C.

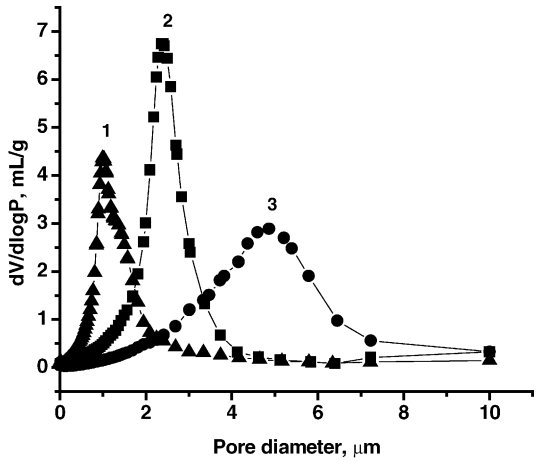

Fig. 3. Pore size distribution profiles determined by mercury intrusion porosimetry for the monoliths used in this study. Median pore size 0.97 (1), 2.30 (2), and 4.07 μm (3). For polymerization conditions see Fig. 1.

would lead to a decrease in pore size and formation of more structured surface manifested by an increase in both back pressure and the surface area. Indeed, the surface area as measured for monolith with a pore size of 0.97 μm by nitrogen adsorption/desorption in the dry state increased from 35.2 to 47.3 m^2/g after coating the monolith with the latex beads. Simultaneously, the back pressure in the column grew from 2.6 to 5.7 MPa at a flow rate of 5 μL/min. This indicates that the layer of latex particles decreases the pore size. The back pressure is controlled by the size of the smallest pores that are also most prone to blocking and even very small particles may significantly decrease the permeability to flow.

The coating of the pore surface with the latex particles can also be visualized using scanning electron microscopy. The micrographs in Fig. 4 clearly demonstrate the change in the structure of surface of the microglobules. Many small particles are attached to the surface. It is worth noting that the surface is covered with individual latex particles rather than with a continuous layer of them. This is due to a limited

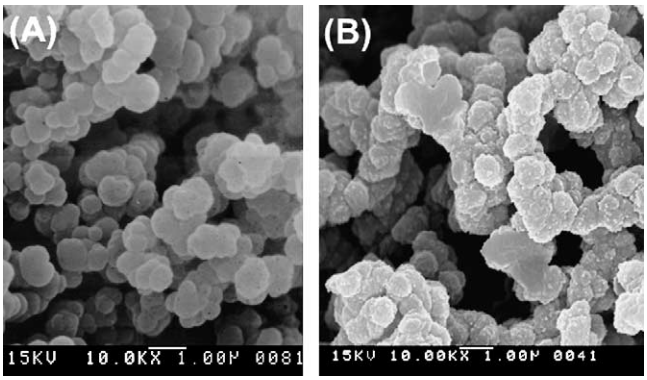

Fig. 4. SEM image of original BuMA–*co*-EDMA–*co*-AMPS monolith (A) and its latex-coated counterpart (B).

percentage of AMPS that can be accommodated in the polymerization mixture. In addition, all the AMPS units are distributed throughout the monolithic matrix and many of them are unreachable for the bulky latex particles. This results in only small proportion of the sulfonic acid functionalities exposed at the accessible pore surface. However, a significant increase of over 30% in the specific surface area after the attachment of the latex particles indicates that the surface coverage is not insignificant.

3.3. Effect of pore size

One of the major advantages of monolithic stationary phases is their low resistance to flow. We have reported very early on that back pressure in monolithic columns decreases with an increase in the pore size [21]. Thus, it would be advantageous to use a monolith with the largest possible pores in order to operate the separation system at high flow velocity and to achieve both fast separations and high throughput. Changing the proportion of 1-propanol in the polymerization mixture enabled the preparation of three monolithic capillary columns with the pore size in a range of 1–4 μm. These monoliths were treated with latex dispersion and their chromatographic performance compared. Fig. 5 clearly shows that the peak resolution significantly deteriorates with the increase in pore size. One of the reasons for this may be the decrease in specific surface area from 35 to 28 to 12 m^2/g for monoliths with a pore size of 4.07, 2.30, and 0.97 μm, respectively. Obviously, the smaller the surface area, the less sulfonic acid functionalities exposed at the surface, and the less immobilized latex particles required for the interactions with the analytes. Since the resistance to flow is high in monoliths with small pores, monolithic column with a median pore diameter of 0.97 μm appears to be optimal and was used for the further studies.

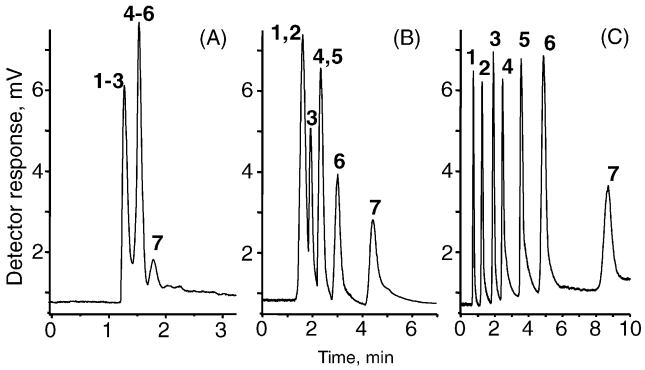

Fig. 5. Effect of pore size on the separation of carbohydrates by anion-exchange chromatography using polymeric monolithic capillary columns. Column size 10 cm ×250 μm i.d., pore size 4.07 (A), 2.30 (B), and 0.97 μm (C) flow rate 13 μL/min mobile phase aqueous ammonium hydroxide 64 mmol/L (pH 12.8). Peaks: D(+)galactose (1), D(+)glucose (2), D(+)xylose (3), D(+)mannose (4), maltose (5), D(−)fructose (6), sucrose (7). Injection volume 100 nL; sample concentration 1 mg/mL.

3.4. Effect of mobile phase

In any ion chromatographic system, increasing the concentration of the competing ion in the mobile phase results in a decrease in retention of the analytes [6]. Thus, a decrease in the concentration of the competing ion is desired to increase both interaction with the stationary phase and retention of separated ions. However, aqueous mobile phases containing inorganic hydroxides are typically used for the chromatographic separation of anions and changing the concentration results in a concomitant change in the pH, which affects the ionization of the analytes. Specifically, high-pH is necessary to ionize the carbohydrates to a certain degree and their separation at a pH value below 11 is not conceivable due to insufficient ionization. On the other hand, at an excessively high-pH, typically more than 13.5 at which all the carbohydrates are fully ionized, the separation is again difficult to achieve. Therefore, we run a series of separations of carbohydrates in the mobile phases in which the concentration of hydroxyl ions varied between 16 to 320 mmol/L translating into pH values between 12.2 and 13.5. As expected for a typical ion chromatography, Fig. 6 confirms that reducing the ammonium hydroxide concentration within this pH range is accompanied with improvements in both peak resolution and retention.

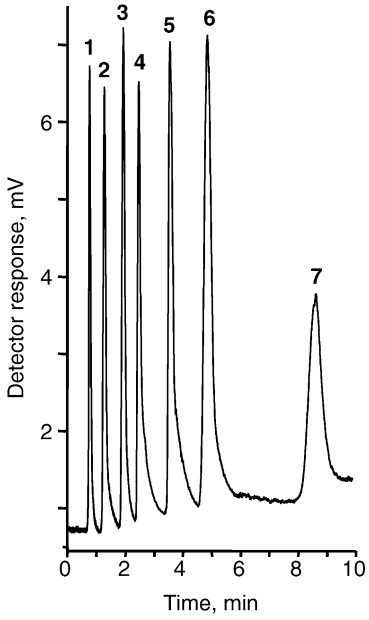

Fig. 7. Separation of a mixture of carbohydrates by anion-exchange chromatography using an optimized latex-coated polymeric monolithic capillary column. Conditions: column size 10 cm ×250 μm i.d., pore size 0.97 μm. Flow rate 13 μL/min mobile phase aqueous ammonium hydroxide 64 mmol/L (pH 12.8). For peak assignment see Fig. 5.

3.5. Chromatographic separations

After optimizing the pore size and composition of the mobile phase, the performance of monolithic capillary columns was demonstrated on the separation of a mixture of saccharides. Evaporative light scattering was used for detection, as it is both suitable to detect underivatized carbohydrates and compatible with flow rates in a range of microliters per minute. This detector operates best with volatile mobile phases. Therefore, ammonium hydroxide was used rather than sodium hydroxide a typical component of mobile phases in ion chromatography. The efficient separation of a mixture of seven carbohydrates was easily achieved in less than 10 min as shown in Fig. 7. The elution order indicates that a simple anion-exchange mechanism is operative in this very efficient separation. Despite the reasonable separation characterized by column efficiency of up to 26 000 plates/m and a good resolution, all of the peaks exhibit tailing. This can be ascribed to the excessive dead volumes and connections of the home-built capillary LC system.

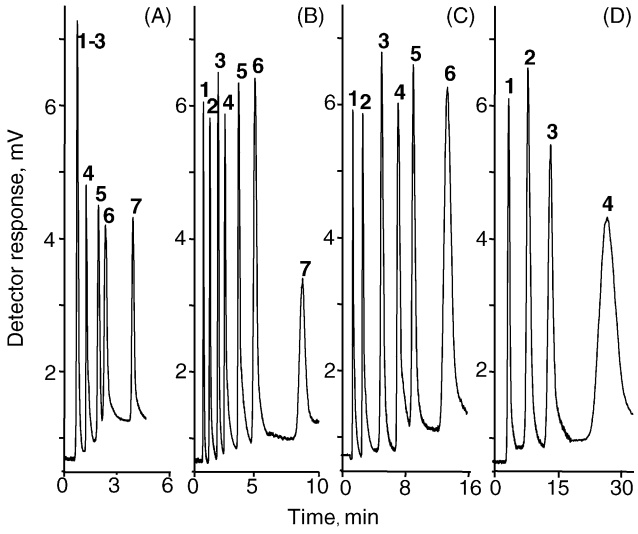

Fig. 6. Effect of the concentration of competing ion and pH on the separation of carbohydrates by anion-exchange chromatography using monolithic capillary columns. Conditions: column size 10 cm ×250 μm i.d., pore size 0.97 μm. Flow rate 13 μL/min. Concentration of ammonium hydroxide in the mobile phase 320 (pH 13.5) (A), 64 (pH 12.8) (B), 32 (pH 12.5) (C), and 16 mmol/L (pH 12.2) (D). For peak assignment see Fig. 5. Injection volume 100 nL; sample concentration 1 mg/mL.

The separation of the sugars derived from the enzymatically processed corn starch using the amylases present in human saliva further demonstrates the analytical potential of this new column. The salivary α-amylases catalyze the hydrolysis of α-1,4 glycosidic linkages in starch to yield maltotriose, maltose and glucose [22]. The chromatogram in Fig. 8 features two peaks that are assigned to glucose and maltose. Similar to saccharose, maltotriose was retained much more strongly then the monosaccharides and therefore did not elute under our experimental conditions.

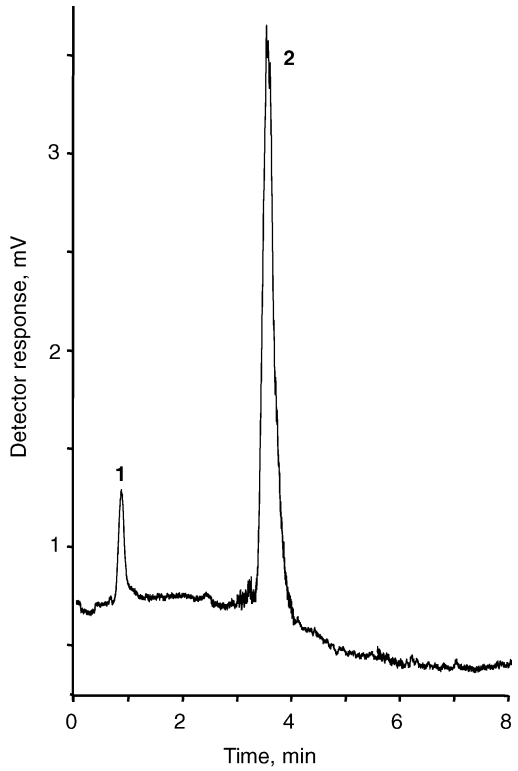

Fig. 8. Anion-exchange chromatography of the carbohydrates of hydrolyzed corn starch using a latex-coated polymeric monolithic capillary column. Conditions: column size 10 cm × 250 μm i.d., pore size 0.97 μm. Flow rate 13 μL/min mobile phase aqueous ammonium hydroxide 64 mmol/L (pH 12.8). The sample was prepared by hydrolysis catalyzed by α-amylases present in human saliva. Peaks: glucose (1), maltose (2). Injection volume 100 nL; sample concentration 1 mg/mL.

4. Conclusions

This preliminary study demonstrates a novel approach to the functionalization of porous polymer monoliths that enables the preparation of an agglomerated ion-exchange stationary phase. Since the monoliths can be readily prepared in a broad range of sizes and a variety of formats [9], this approach opens new avenues to an alternative and perhaps more flexible preparation technique affording stationary phases suitable for the separation of a variety of analytes using ion-exchange chromatographic mode. Although only a model mixture of simple carbohydrates was used in this study, the results indicate, that the highly porous structure typical of the monoliths enables again to achieve rather fast separation without losing the efficiency. Our work also demonstrates a viable approach to the rapid and efficient determination of carbohydrates in a miniaturized capillary format using the mobile phase that is directly compatible with mass spectrometric detection. This should find a positive resonance in the areas of glycobiology and glycotechnology where the demand for such miniaturized separation systems is most apparent.

Our ongoing research targets improvements in the peak shape that are expected to occur as a result of total reconstruction of our chromatographic system as well as in the further increase in the pore surface coverage. Instead of copolymerizing we plan to use surface grafting with AMPS we have developed previously [16] which is expected to result in higher coverage of the pore surface with sulfonic acid functionalities and consequently with the latex particles.

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