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Methacrylate monolithic capillary columns for gradient peptide separations

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ABSTRACT

For the separation of peptides with gradient-elution liquid chromatography a poly(butyl methacrylate-*co*-ethylene dimethacrylate) (BMA) monolithic capillary column was prepared and tested. The conditional peak capacity was used as a metric for the performance of this column, which was compared with a capillary column packed with C18-modified silica particles. The retention of the peptides was found to be smaller on the BMA column than on the particulate C18 column. To obtain the same retention in isocratic elution an approximately 15% (v/v) lower acetonitrile concentration had to be used in the mobile phase. The retention window in gradient elution was correspondingly smaller with the BMA column. The relation between peak width and retention under gradient conditions was studied in detail. It was found that in shallow gradients, with gradient times of 30 min and more, the peak widths of the least retained compounds are strongly increased with the BMA column. This was attributed to the fact that these compounds migrate and elute with an unfavorable high retention factor. More retained compounds are eluted later in the gradient, but with a lower effective retention factor. With shallow gradients the peak capacity of the BMA column (≈90) was clearly lower than that of a conventional packed column (≈150). On the other hand, with steep gradients, when components elute with a low effective retention factor, the performance of the BMA column is relatively good. With a gradient time of 15 min similar peak widths and thus similar peak capacities (≈75) were found for the packed and the monolithic column. Two strategies were investigated to obtain higher peak capacities with methacrylate monolithic columns. The use of lauryl methacrylate (LMA) instead of butyl methacrylate (BMA) gave an increase in retention and narrower peaks for early eluting peptides. The peak capacity of the LMA column was ≈125 in a 60 min gradient. Another approach was to use a longer BMA column which resulted in a peak capacity of ≈135 could be obtained in 60 min.

1. Introduction

The interest in micro-total-analysis systems (μTAS) or lab-on-a-chip technology is still growing. This is mainly due to the expected superior performance of micro-fabricated devices in terms of speed and sample throughput compared to analytical equipment of conventional size. Also, micromachining technologies provide improved possibilities for the creation of integrated complex systems, for example for two-dimensional separations (in time or in space) [1–6]. These microfluidic devices may be employed for the separation of complex mixtures, such as peptides in a tryptic digest, when a one-dimensional separation does not provide the necessary resolving power (peak capacity). Comprehensive multi-dimensional separation systems are increasingly applied in new application areas of analytical chemistry (proteomics, metabolomics), preferably interfaced with high-resolution mass spectrometers for detection and identification.

The peak capacity of a two-dimensional separation system is ideally the product of the peak capacities of the individual dimensions. However, the entire peak capacity can only be used if we are able to realize orthogonal dimensions, i.e. when the mechanisms by which the analytes are separated are uncorrelated. Completely orthogonal two-dimensional separations are hardly ever encountered in practice, but we should pursue a highest possible degree of orthogonality by carefully selecting both dimensions, in such a way that they reflect the variation in dif-

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ferent physico-chemical properties of the sample components as much as possible. Good examples are the combinations of strong cation-exchange chromatography and reversed-phase liquid chromatography (SCX × RPLC) for separating peptides [7] and of RPLC and size-exclusion chromatography (RPLC × SEC) for separating synthetic polymers [8].

In chip-based separations many different separation mechanisms have been applied, *viz.* electro-driven separations, such as capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC) and isoelectric focusing (IEF) [9–11], topographic separations in nano-structured channels [12–19], and various modes of liquid chromatography in channels packed with particles [20,21] or monolithic stationary phases [22–25]. For application in microfluidic separation devices methacrylate monoliths are of special interest, for several reasons. Their main advantage is the possibility of *in situ* production of methacrylate-based stationary phases by means of a single-step UV-initiated polymerization reaction, which makes it possible to precisely determine the position of the retentive phase in the microfluidic device. The reaction mixture can be introduced into the channel as a liquid, which is much more convenient than packing particles. A second advantage concerns the high permeability of methacrylate monolithic columns, resulting in low back pressures. Most microfluidic devices cannot stand high pressures.

Methacrylate monolithic stationary phases have been introduced by Svec and Frechet over a decade ago [26] and since that time much effort has been put into the study and development of these materials. This has resulted in a wide range of available materials with different monomers incorporated in the polymer to perform various modes of liquid chromatography [27–33] and in an increased understanding of the factors determining the analytical performance [34–37].

Previous work in our group has led to the conclusion that in isocratic separations the efficiency of methacrylate monolithic capillary columns is high for unretained compounds, but that it decreases with retention. This was observed for different classes of compounds, albeit to varying extents. In contrast, Eeltink et al. [32] and Le Gac et al. [24] showed fast and efficient separations of peptide mixtures under gradient conditions, using a lauryl methacrylate (LMA) monolith. An explanation for this difference in behavior of a methacrylate column in isocratic or gradient separations may be found in the type of application. The peptides present in tryptic digests show a very strong dependence of their retention factors (k) on the volume fraction of organic modifier in the mobile phase. This means that minor changes in the composition of the mobile phase can cause a peptide to be either almost indefinitely retained or virtually unretained. This behavior, referred to as an on/off mechanism, may provide an explanation for the good results presented for gradient separations. In the study described in the present paper we have used a novel approach to investigate the retention and efficiency characteristics of a butyl methacrylate monolithic column in the isocratic and gradient modes. Moreover, we have compared the results obtained with that of a commercially available capillary column packed with C18-modified silica particles, which is the most common stationary phase for peptide separations, to put the performance of the methacrylate monolith in perspective. Also we optimized the conditional peak capacity in relation to the gradient duration for both types of columns, for a specific mixture of peptides that is thought to be representative for the components of a typical tryptic digest [40]. For this optimization we evaluated the retention behavior of the peptides on both columns, which determines the size of the retention window, and the relation between retention and peak width in gradient separations.

Table 1
Compound name, molecular weight and m/z value used for MS detection of model peptides

Peptide	M_r	m/z
Gly-Phe	222.24	223.24 (1+)
Neurotensin fragment 1–8	1030.13	516.06 (2+)
Phe–Phe	312.36	313.36 (1+)
Luteinizing hormone releasing hormone	1182.29	592.15 (2+)
Angiotensin 2	1046.18	524.09 (2+)
[Val^5]-angiotensin 1	1282.45	642.23 (2+)
Substance P	1347.63	674.82 (2+)
Renin substrate	1759.01	880.51 (2+)
Momany peptide	770.88	771.88 (1+)
Insulin chain B oxidized	3495.89	874.97 (4+)
Melittin	2846.46	712.62 (4+)

2. Experimental

2.1. Materials and reagents

Fused-silica capillaries with a UV-transparent coating were purchased from Polymicro Technologies (Phoenix, AZ, USA). The peptides used as model compounds (Table 1), as well as the ingredients of the polymerization mixture, butyl methacrylate (99%), ethylene dimethacrylate (98%, EDMA), lauryl methacrylate (99%), 1,4-butanediol (99%), azobisisobutyronitrile (98% AIBN) and 3-(trimethoxysilyl)propyl methacrylate (99%, γ-MAPS), were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Recombinant chlorite dismutase (a gift from Karlstad University Department of Chemistry) was treated with trypsin for 20 h at 37 °C. The laboratory-prepared BMA monolithic columns were compared with an Agilent capillary column of 15 cm length and an I.D. of 75 μm, packed with 3.5-μm Zorbax 300SB-C18 particles.

Other chemicals were obtained from standard suppliers and used as received.

2.2. Preparation of monolithic columns

Butyl methacrylate monolithic columns were prepared in fused-silica capillaries of 75 μm internal diameter after vinylization of the inner wall to ensure covalent anchoring of the monolith to the capillary wall. Following the procedure described by Yu et al. [38], capillaries were rinsed with acetone, flushed with 0.2 M sodium hydroxide for 30 min and washed with deionized water, again flushed for 30 min with 0.2 M hydrochloric acid and rinsed with ethanol. Next, a 20% solution of 3-(trimethoxysilyl)propyl methacrylate in ethanol at pH 5 was flushed through the capillary at a rate of 0.25 μL/min for 1 h using a syringe pump (KdScientific, New Hope, PA, USA). After this, the capillary was flushed with acetone and dried overnight by a stream of compressed air. The polymerization mixture consisted of 24% BMA or LMA, 16% EDMA, 33.8% 1-propanol, 25.8% 1,4-butanediol and 0.4% AIBN (all w/w) [39]. After injecting the mixture into the capillaries, the ends were sealed with pieces of septum. A UV-Crosslinker (Spectroline, Westbury, NY, USA) was used to irradiate the capillaries with an intensity of approximately 3 mW/cm^2 for 50 min at 254 nm. Before use, the monolith-filled capillaries were flushed extensively with acetonitrile (ACN) and cut to a length of 25 cm.

2.3. Instrumentation and chromatographic conditions

Isocratic and gradient experiments were performed using an Agilent 1100 series NanoLC system interfaced to an ion-trap mass spectrometer via an orthogonal nanospray ion source (Agilent Technologies, Waldbronn, Germany) with a picotip emitter needle of 3 cm length and 8 μm I.D. (New Objective, Woburn, MA, USA). Elec-