

Fast Protein and Peptide Separations Using Monolithic Nanocolumns and Capillary Columns

INTRODUCTION

Polymeric monolithic stationary phases offer an alternative to the classical microparticulate sorbents, bringing important advantages to sample analysis. In contrast to the traditional stationary phases that consist of packed particles, the monolithic separation medium is made of a continuous, rigid polymeric rod with a porous structure. The lack of intraparticle void volume improves mass transfer and separation efficiency, which allows for very fast separations of biopolymers.^{1,2}



Figure 1. Monolithic column in protective housing.

INSTRUMENTATION

All experiments were performed on the UltiMate™ Plus Nano and Capillary LC System equipped with a special 3-nL UV flow cell, and the FAMOS™ Micro Autosampler. The Monolithic nanocolumn (100- μm i.d. \times 5 cm) or capillary column (200- μm i.d. \times 5 cm), consisting of PS-DVB (polystyrene-divinylbenzene polymer), was thermostatted at 60 °C, using the UltiMate column oven. UV detection was performed at 214 nm, and the flow rate was either 1 $\mu\text{L}/\text{min}$ (100- μm i.d.) or 2.5 $\mu\text{L}/\text{min}$ (200- μm i.d.). For LC-MS, the system was coupled on-line to the esquire3000 plus (Bruker Daltonics).

HIGH-RESOLUTION PROTEIN AND PEPTIDE SEPARATIONS

Figure 2 shows the separation of a tryptic digest of cytochrome c on a 100- μm i.d. Monolithic LC nanocolumn. A gradient from 0-45% acetonitrile in acidified water (0.04% TFA) is performed in 8 min, resulting in a fast separation of each peptide with baseline resolution.

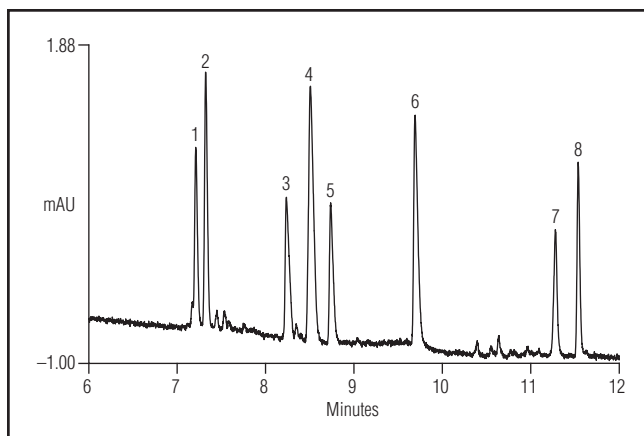


Figure 2. Nano LC separation of cytochrome *c* sample (800 fmol/ μ L). Injected amount 0.3 μ L.

Table 1. Peak Width at Half Height (PWHH) for Peptides Separated on a Monolithic Capillary Column

Peptide	Retention Time min	PWHH s
1. Bradykinin fragment 1–5	3.3	3.5
2. Vasopressin [Arg ⁸]	3.8	1.6
3. Methionine enkephalin	4.0	1.9
4. Leucine enkephalin	4.4	2.3
5. Oxytocin	4.6	1.6
6. Bradykinin	4.9	2.5
7. LHRH	5.1	1.9
8. Bombesin	6.3	2.0
9. Substance P	6.4	2.6

Figure 3 shows the separation of a test mixture consisting of nine peptides on a 200- μ m i.d. Monolithic capillary column. A gradient from 0–25% acetonitrile in water, 0.05% trifluoroacetic acid (TFA) is performed in 7 min, resulting in a fast baseline separation of all peptides. Peak widths at half height (PWHH) of only 1.6–3.5 s illustrate the fast separations that are achievable using a Monolithic capillary column (see Table 1). Under isocratic conditions, efficiencies up to 250,000 plates/m are achieved.

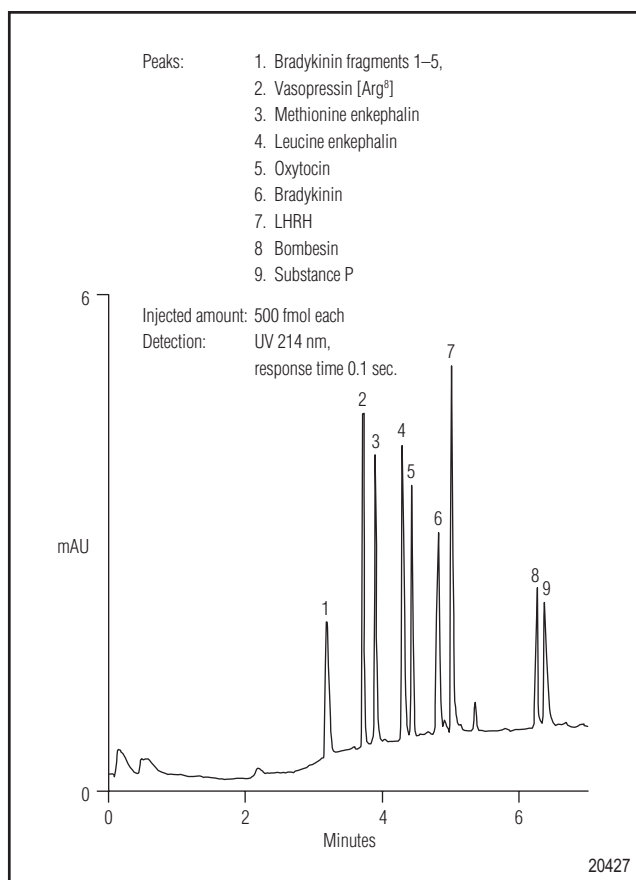


Figure 3. Separation of a peptide test mixture.

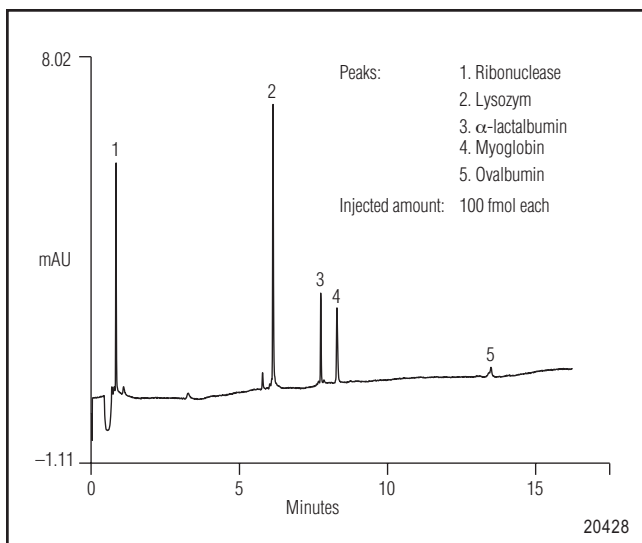


Figure 4. Separation of proteins.

Aside from the analysis of peptides, polypeptides, and protein digests, interest is increasing in proteomics for the analysis of intact proteins. Therefore, the same 200- μm i.d. Monolithic capillary columns have been evaluated for the separation of protein mixtures. Figure 4 shows the separation of a protein mixture using a gradient of 20–50% acetonitrile in water, 0.05% TFA, in 15 min. Similar separation efficiencies are achieved for the peptides, illustrating the excellent performance of Monolithic columns for both peptides and proteins. Figure 5 shows a closeup of the chromatogram of peaks 3 and 4, illustrating symmetrical peak shapes and PWHHs of only a few seconds.

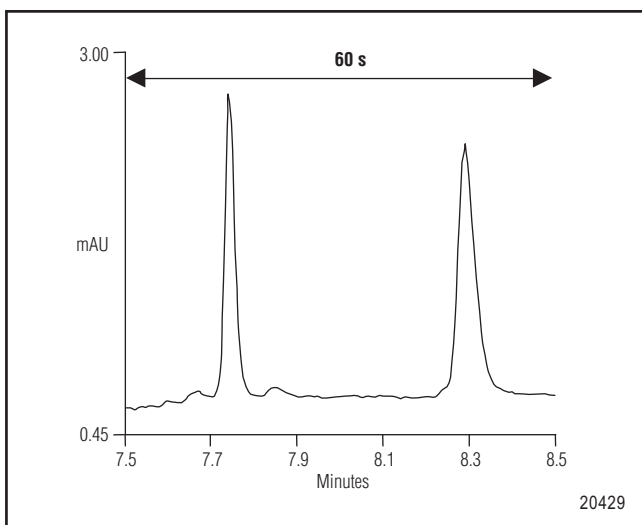


Figure 5. Closeup of the separation of proteins 3 and 4.

TFA VS FORMIC ACID

For highly sensitive LC-MS applications using nano-electrospray, weaker acids—such as formic acid (FA)—are often preferred over the stronger TFA as mobile phase additives to reduce the discrimination effect. Figure 6 shows a comparison using both additives for the separation of the same peptide test mixture. Independent of the mobile phase additive (i.e., TFA or formic acid), excellent separations are obtained. Using formic acid as an ion pair, PWHH increased only marginally and the peak height decreased by only 20–30%.

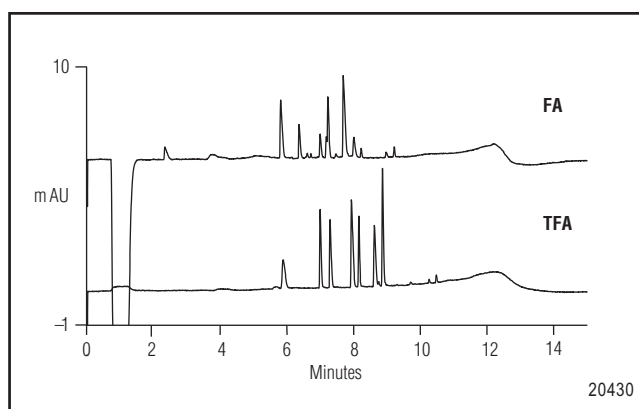


Figure 6. Separation of peptide test mixture (peptides 1–9, see Figure 2) on a Monolithic capillary column with 0.1% formic acid (FA) and 0.05% TFA as mobile phase additives.

ON-LINE COUPLING TO ESI-MS

To illustrate the use of Monolithic capillary columns in LC/MS, Figure 7 shows the separation of a protein digest mixture. The mixture consists of cytochrome c, lysozyme, alcohol dehydrogenase, BSA, apotransferrin, and β -galactosidase with a molecular weight ranging from 11 to 135 kDa and digested with trypsin. The gradient consists of (A) 0.1% formic acid, and (B) 80% acetonitrile, 0.08% formic acid, from 0 to 50% B in 25 min. Figure 6A shows the base peak chromatogram (BPC), illustrating the high peak capacity of the Monolithic column for the separation of complex samples. Figure 6B shows a closeup of the BPC between 16.0 and 18.0 min with two minor tryptic peptides eluting at 17.0–17.2 min. From the tryptic peptide eluting at 17.0 min, the MS-MS spectrum is shown in Figure 6C with the corresponding Mascot® database search (Figure 6D). This peptide has the sequence GLVLIAFSQYLQCPFDEHVK and could be unambiguously identified as a BSA fragment.

CONCLUSION

Monolithic nanocolumns and capillary (polymer-based) columns show excellent separation performance. The same column can be used for both protein and peptide separations. To achieve separation efficiencies of up to 250,000 plates/m, the use of a dedicated nano HPLC system with zero dead volumes is required. Using 5-cm columns results in very fast protein and peptide separations with a PWHH of only a few seconds. The monolithic structure is also advantageous because the very robust column bed results in zero voiding and a superior column lifetime. Coupled to MS, Monolithic nanocolumns and capillary columns allow for high-throughput and sensitive analysis for peptides and proteins, independent of the mobile phase additive (TFA or FA).

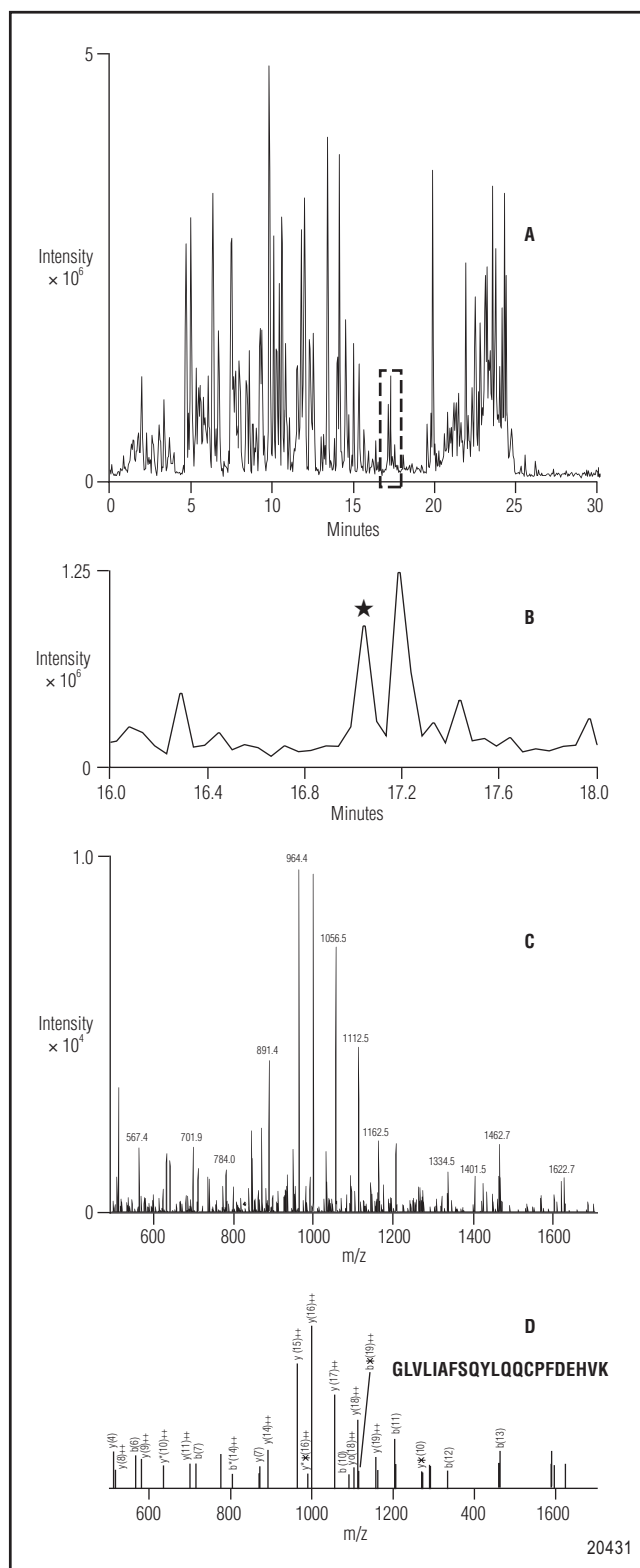


Figure 7. (A) LC/MS using a Monolithic capillary column (PS-DVB based). BPC of digested protein mixture. Injected amount 100 fmol. Dashed lines represent zoom-in of Figure 6D. (B) Zoom-in of BPC with an arbitrarily chosen tryptic peptide (★) used for MS and MS/MS investigation. (C) MS/MS of tryptic peptide, eluting at 17.0 min with m/z 832.2. (D) Mascot search.

REFERENCES

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2. Walcher, W.; Oberacher, H.; Troiani, S.; Holzl, G.; Oefner, P.; Zolla, L.; Huber, C. G. Monolithic Capillary Columns for Liquid Chromatography: Electrospray Ionization Mass Spectrometry in Proteomic and Genomic Research. *J. Chromatogr., B* **2002**, *782*, 111–125.



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