Fast Protein/Peptide Separations Using Monolithic Capillary Columns

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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, which consist of packed particles, the monolithic separation medium is made of a continuous, rigid polymeric rod with a porous structure. The lack of intraparticular void volume improves mass transfer and separation efficiency, allowing for fast separations of biopolymers.1,2

Instrumentation
All experiments were performed on the UltiMate™ Capillary/Nano LC System equipped with a special 3 nL UV flowcell, and the FAMS™ Micro Autosampler. The monolithic capillary column, 200 µm i.d. x 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, flow-rate was set at 2.5 µL/min (gradient mode). For LC–MS the system was coupled on-line to the Esquire™ 3000+, using a Bruker NanoSpray source. Needle: 50 mm i.d., 30 mm i.d. tip (New Objective).

High-Resolution Protein and Peptide Separations
In Figure 2 the separation of a test mixture consisting of nine peptides is shown. A gradient from 0–25% acetonitrile in water, 0.05% TFA is performed in 7 min resulting in a fast separation of...

Table 1: Peak width at half height (PWHH) for peptides separated on a monolithic capillary column.

<table>
<thead>
<tr>
<th>Peptide #</th>
<th>Retention time min</th>
<th>PWHH s</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Bradykinin fragment 1–5</td>
<td>3.3</td>
<td>3.5</td>
</tr>
<tr>
<td>2. Vasopressin [Arg8]</td>
<td>3.8</td>
<td>1.6</td>
</tr>
<tr>
<td>3. Methionin enkephalin</td>
<td>4.0</td>
<td>1.9</td>
</tr>
<tr>
<td>4. Leucin enkephalin</td>
<td>4.4</td>
<td>2.3</td>
</tr>
<tr>
<td>5. Oxytocin</td>
<td>4.6</td>
<td>1.6</td>
</tr>
<tr>
<td>6. Bradykinin</td>
<td>4.9</td>
<td>2.5</td>
</tr>
<tr>
<td>7. LHRH</td>
<td>5.1</td>
<td>1.9</td>
</tr>
<tr>
<td>8. Bombesin</td>
<td>5.1</td>
<td>1.9</td>
</tr>
<tr>
<td>9. Substance P</td>
<td>6.4</td>
<td>2.6</td>
</tr>
</tbody>
</table>

Peaks: 1 = bradykinin fragments 1–5, 2 = vasopressin [Arg8], 3 = methionin enkephalin, 4 = leucin enkephalin, 5 = oxytocin, 6 = bradykinin, 7 = LHRH, 8 = bombesin, 9 = substance P.
each peptide with baseline/baseline resolution. Peak widths at half height (PWHH) of only 1.6–3.5 s illustrate the fast separations that are achievable using monolithic capillary columns (see Table 1). Under isocratic conditions efficiencies of up to 250 000 plates per metre are achieved.

Beside the analysis of peptides, polypeptides and protein digests there is an increasing interest for the analysis of intact proteins in Proteomics. Therefore the same monolithic columns have been evaluated for the separation of protein mixtures. Figure 3 shows the separation of a protein mixture using a gradient of 20–50% ACN in water, 0.05% TFA, in 15 min. Similar separation efficiencies are achieved as for the peptides illustrating the excellent performance of monolithic columns for both peptides and proteins. In Figure 4 a zoom-in of the chromatogram of peak #3 and #4 is shown illustrating symmetrical peak shape and PWHH of a few seconds only.

TFA vs Formic Acid?
For high sensitive LC–MS applications such as NanoElectroSpray the use of weaker acids such as formic acid is often preferred over the stronger TFA as mobile phase additive in order to reduce the

Peaks: 1 = ribonuclease, 2 = lysozyme, 3 = lactalbumin, 4 = myoglobin, 5 = ovalbumin.

Figure 3: Separation of proteins. Injected amount 100 fmol each.

Figure 4: Zoom-in of separation of proteins 3 and 4.

Figure 5: Separation of peptide test mixture (peptides 1–7) on monolithic capillary column with 0.1% Formic Acid (FA) and 0.05% TFA as mobile phase additive.

Figure 6(a): LC/MS using monolithic capillary column (PS-DVB based). BPC of digested protein mixture. Injected amount 100 fmol. Dashed lines zoom-in of Figure 6(d).
discrimination effect. Figure 5 shows the comparison using both additives for the separation of the peptide test mixture. Independent of the mobile phase additive (i.e., TFA or formic acid) an excellent separation has been obtained. Using formic acid as ion pair PWHH increase only marginally and the peak height decreased by only 20–30%.

**On-line coupling to ESI-MS**

To illustrate the use of monolithic capillary columns in LC–MS, the separation of a protein digest mixture is shown in Figure 6. The mixture consist of cytochrome C, lysozyme, alcohol dehydrogenase, BSA, Apo-transferrin and β-Galactosidase with a molecular weight ranging from 11–135 kDa and digested with trypsin. The gradient consist of A) 0.1% formic acid and B) 80% ACN, 0.08% formic acid, from 0 to 50% B in 25 min. In Figure 6(a) the base peak chromatogram (BPC) is shown illustrating the tremendous peak capacity of the monolithic column for the separation of complex samples. In Figure 6(b) a zoom of BPC is shown between 16.0 and 18.0 min with two minor tryptic peptides eluting at 17.0 and 17.2 min. From the tryptic peptide eluting at 17.0 min, the MS/MS spectrum is shown in Figure 6(c) with the corresponding MASCOT database search (Figure 6(d)). This peptide has the sequence GLVLIAFSQYLQCPEDEHVK, and could be unambiguously identified as a BSA fragment.

**Conclusions**

Monolithic capillary columns (polymer-based) show excellent separation performance. The same column can be used for both protein and peptide separations. In order to achieve separation efficiencies of up to 250 000 plates/metre the use of a dedicated Nano HPLC system with zero-dead volumes is required. Using 5 cm columns results in very fast protein/peptide separations with peak width at half height (PWHH) of a few seconds only. 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 capillary columns allow for high-throughput and sensitive analysis for peptides and proteins independent of the mobile phase additive (TFA or FA).

**References:**