Peptide Separations by Microbore Reversed-Phase HPLC

Application Note

Bioscience

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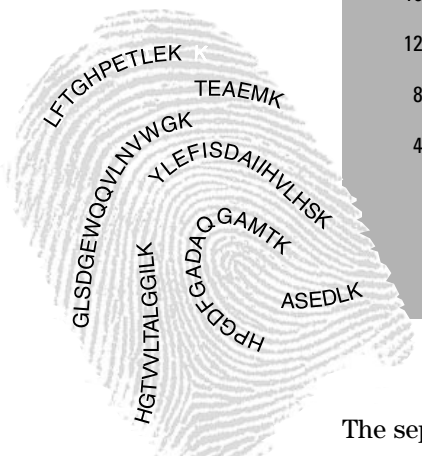

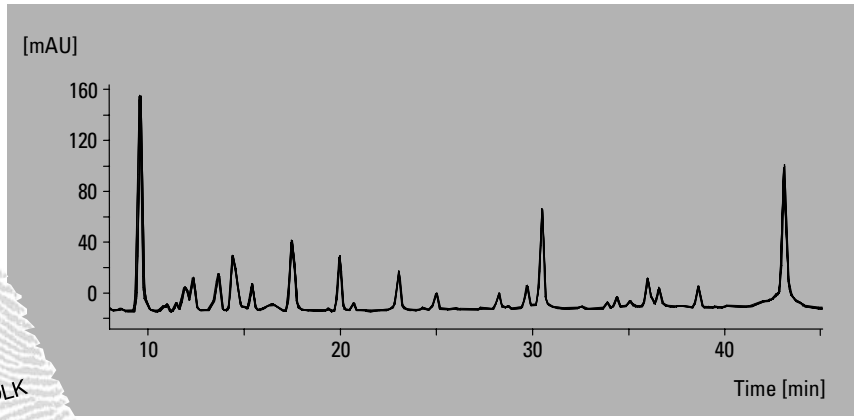

The separation of peptide mixtures on 1-mm internal diameter (id) reversed-phase columns—to characterize proteins or to isolate peptides for further studies—places stringent demands on the HPLC system. For routine application of shallow gradients the HPLC system must mix and deliver solvents precisely, have a small delay volume and provide excellent column thermostatting. The UV-detection of peptides in the subpicomole range requires a low-noise detector (preferably based on a diode array) as well as minimum external band-broadening. In this study highly-reproducible peptide maps were produced and the detectability of peptides was tested. Further, as a new aspect, rapid peptide separations on standard reversed-phase packing material were performed.

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Introduction

Separation of peptide mixtures by reversed-phase HPLC on microbore columns (1-mm id) is a powerful method for the characterization of proteins. After enzymatic or chemical cleavage of a protein the resulting peptides are separated according to their relative surface hydrophobicity. The peptides are detected spectrophotometrically in the UV range at wavelengths where the peptide bonds absorb (α = 205–220 nm) or where the aromatic side chains of tyrosine, phenylalanine and tryptophan absorb (α_{max} = 250–290 nm). The resulting chromatographic pattern—the peptide map—is a unique feature, a fingerprint of a protein analysed under identical conditions. Peptide mapping has therefore been used extensively in the quality control of recombinant proteins for human therapeutic use. Of similar importance is the separation and isolation of peptides for further examination such as sequence analysis or mass determination.

For reliable peptide separations an HPLC system is required in which all modules provide high levels of performance. Factors ranging from proper solvent degassing to low flow-cell volume all influence the qualitative and quantitative aspects of the chromatography. Table 1 summarizes the beneficial influence that a well-performing HPLC system can have on highly-qualitative microbore HPLC.

The chromatographic parameters for peptide mapping on various diameter columns have been described in previous studies.[1,2] In this study we describe the instrumental prerequisites for successful peptide mapping on 1-mm id columns and show results using an Agilent 1100 Series system for HPLC.

Theory of chromatographic resolution

For a given solvent system and column, the resolution between peaks in a peptide separation depends on:

- the gradient slope,
- the column temperature, and
- external band-broadening in the HPLC system.

Gradient slope

The gradient slope has a significant impact on resolution and is not only defined by the time program of the solvent composition but also by the delay volume of the HPLC system relative to the column volume. The delay volume comprises the volume of solvent delivered between the mixing point of the gradient solvents and the top of the column—capillaries, mixer, and injector all contribute to delay volume.

	Solvent Delivery System	Autosampler	Column Thermostat	Detector	External Volumes
Chromatographic Resolution	Small delay volume for peaks eluting at low %B, and defined, linear gradients	Low sample path volumes for peaks eluting at low %B Low delay volume	Low sample path volumes for peaks eluting at low %B	Low volume cell Short, low volume heat exchanger and connection capillaries	Short, low volume connection capillaries
Retention-Time Reproducibility	Flow resolution in low nl range		Good temperature precision High ambient rejection		
Area Reproducibility	Flow resolution in µl/nl range	High precision of small injection volumes		Low noise Low drift Small refractive index effects	
Detectability	Excellent mixing properties		High temperature precision	Low noise Low drift Small refractive index effects	
Analysis Time	Small delay volume	Small delay volume			

Table 1
Quality parameters and instrumental demands for high-performance peptide mapping on microbore columns

A 1 × 250 mm column packed with 5-µm reversed-phase material has a dead volume—that is, column volume minus volume of the packing material—of about 140 µl. To produce relatively undistorted gradient profiles the system delay volume should not exceed the column volume by more than a factor of 1–2, in this example 140–280 µl. When the ratio of delay volume to column volume increases, the gradient becomes more distorted (see figure 1) and the programmed profile is *washed out*, especially in the early part of the gradient. The actual slope is lower than the programmed slope. This can cause selectivity changes as well as broader peaks. Whereas broader peaks are generally disadvantageous, the change in selectivity can lead to better or worse resolution. A system with a small delay volume can perform steep or flat gradients and can be optimized for maximum resolution. In contrast, a system with a large delay volume does not allow defined slopes at the beginning of the gradient and has therefore less flexibility for optimization of resolution.

However, an adequate mixing volume is needed to maintain low noise levels (1–2 × 10^{-5} AU) for optimum detectability. Therefore pumps providing adequate mixing at low delay volumes are preferred—for highest detectability a low volume mixer should be added for maximum baseline smoothness.

Column temperature

Column temperatures between 30 and 50 °C usually provide best resolution. Higher temperatures give more plates but often reduce selectivity. The optimum temperature must be found empirically.

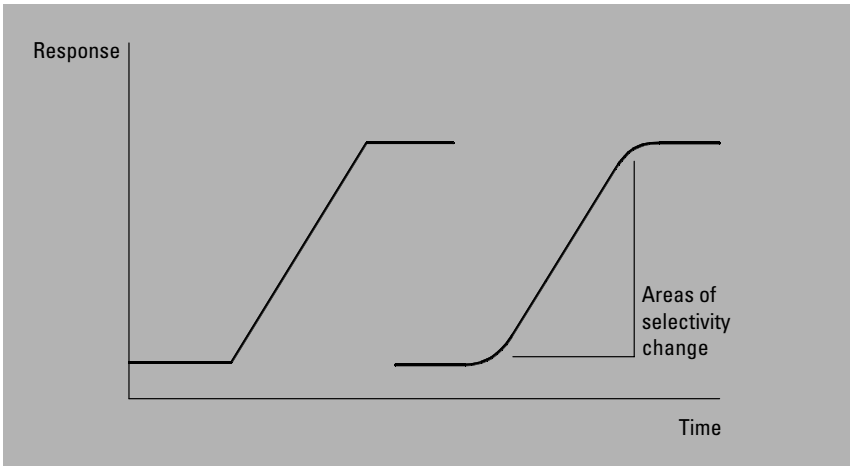


Figure 1
Effect of delay volume on gradient shape

External bandbroadening

Microbore columns generate small peak volumes. To maintain the plate number of the column, the external volumes (connecting capillaries, heat exchangers and flow cells) must be as small as possible. Small flow cells are often a compromise in terms of volume, path length and light throughput. Small volumes result in better dispersion (increased resolution). Short path lengths mean less light throughput (increased noise, decreased sensitivity and detectability) as well as more severe refractive index effects (decreased detectability).

Experimental

Horse skeletal muscle myoglobin, TPCK-treated trypsin, dithiothreitol and iodoacetic acid were obtained from Sigma (Deisenhofen, Germany). The HPLC standard peptide mixture from Sigma contained Gly-Tyr, Val-Tyr-Val, Met-enkephalin and angiotensin II (w/w ratio 1:4:4:4:4). HPLC-grade acetonitrile was obtained from Baker Analyzed (Deventer, The Netherlands). HPLC-grade trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL, USA). All other chemicals were from Fluka (Buchs, Switzerland). Distilled and deionized water was obtained from a Millipore Milli-Q water system (Eschborn, Germany).

Myoglobin was dissolved in 6-M guanidine hydrochloride, 50-mM Tris, pH 8, and incubated for 30 min at room temperature. After adding dithiothreitol (100-fold molar excess) and incubation at room temperature for 30 min, the solution was mixed with iodoacetic acid (100-fold molar excess) and allowed to stand for 60 min in the dark. Alkylation was stopped by adding excess dithiothreitol. Volumes of 100-mM ammonium hydrogen carbonate buffer at pH 8 were added to decrease the guanidine hydrochloride concentration to 1 M. The final protein concentration was 3.3 mg/ml. For digestion, trypsin was added to the protein solution (25:1 w/w) which was incubated for 22 hours at 37 °C. The reaction was stopped by reducing the pH to below 4 with TFA.

Peptide separations were performed using an Agilent 1100 Series system controlled through Agilent ChemStation software for LC. The system comprised an Agilent 1100 Series vacuum degasser, high-pressure binary pump, autosampler, thermostatted column compartment and diode-array detector. To reduce the extra-column volume all connecting tubings were 0.12-mm id. The columns were obtained from MZ-Analysentechnik (Mainz, Germany). These were packed with Vydac 218TP (5 µm, 300 Å, C18). All other chromatographic conditions are given in the figures.

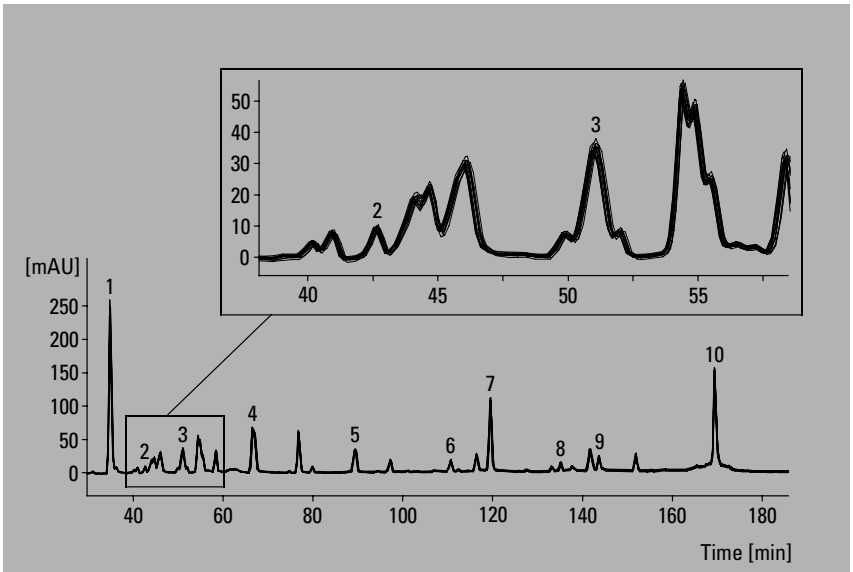
Results and discussion

Retention-time and area reproducibility

Peptide mapping is a versatile method for the quality control of recombinant proteins or biopharmaceutical drugs. In practice, the chromatographic result is used as a *fingerprint* and compared to the data of a control analysis under identical conditions. Minute differences between the chromatograms must be identified and, in some cases, characterized further. In this application area the chromatography must be highly stable and retention-time reproducibility is the most important parameter.

Figure 2 shows the results of sequential peptide mapping with shallow gradients on a 1-mm id column. The excellent retention-time reproducibilitiy was primarily due to the properties of the solvent delivery system and the column thermostat, whereas the injector was responsible for reproducible peak areas (see figure 3).

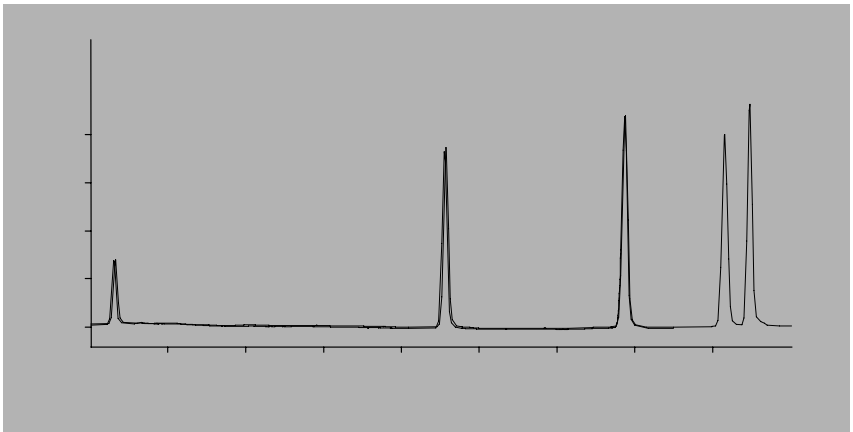
A vacuum degasser continuously removed air from the mobile phase, resulting in a substantial decrease in gradient disturbances. In contrast to helium sparging, degassing by application of vacuum to the PTFE solvent tubings did not evaporate TFA out of the mobile phase. The TFA concentration therefore remained unaffected even after prolonged solvent use.



Peak No.	1	2	3	4	5	6	7	8	9	10
Mean RT	34.82	42.65	51.03	66.58	89.40	110.64	119.53	135.19	143.63	169.36
SD RT	0.059	0.058	0.075	0.089	0.088	0.058	0.085	0.081	0.059	0.071
RSD RT	0.17	0.14	0.15	0.13	0.10	0.05	0.07	0.06	0.04	0.04

Column	1 × 250 mm filled with Vydac TP218
Solvent A	0.05 % TFA in water
Solvent B	0.043 % TFA in acetonitrile
Static mixer	Without mixer
Flow rate	50 µl/min
Temperature	40 °C
Gradient	0 min, 1 %B 200 min, 51 %B 210 min, 70 %B 210.01 min, 70 %B, 0.12 ml/min 215 min, 98 %B, 0.12 ml/min 217 min, 98 %B, 0.12 ml/min 220 min, 1 %B, 0.12 ml/min 240 min, 1 %B, 0.12 ml/min 240.01 min, 1 %B, 0.05 ml/min 250 min, 1 %B
Sample	Tryptic digest of myoglobin 3.3 mg/ml
Injection	1.1 µl, 190 pmol
Detection	214/8 nm, reference 450/80 nm
Flow cell	1.7 µl volume, 6 mm path length
Slit width	4 nm

Figure 2
Overlay of 14 consecutive runs of a tryptic digest of myoglobin separated on a 1-mm id column



Peak Number	1	2	3	4	5
Mean RT	13.14	55.55	78.78	91.63	94.89
SD RT	0.175	0.130	0.101	0.101	0.097
RSD RT	1.33	0.23	0.13	0.11	0.10
Mean Area	248.76	838.60	1058.82	992.95	977.97
SD Area	1.484	4.698	4.092	3.948	2.357
RSD Area	0.60	0.56	0.39	0.40	0.24

Column	1 × 250 mm filled with Vydac TP218
Solvent A	0.05 % TFA in water
Solvent B	0.043 % TFA in acetonitrile
Static mixer	With mixer
Flow rate	50 µl/min
Temperature	40 °C
Gradient	0 min, 1 %B 200 min, 51 %B 210 min, 70 %B 210.01 min, 70 %B, 0.12 ml/min 215 min, 98 %B, 0.12 ml/min 217 min, 98 %B, 0.12 ml/min 220 min, 1 %B, 0.12 ml/min 240 min, 1 %B, 0.12 ml/min 240.01 min, 1 %B, 0.05 ml/min 250 min, 1 %B
Sample	Sigma HPLC peptide standard mixture, 25 ng/µl
Injection	2 µl, 47–131 pmol of analytes
Detection	214/8 nm, reference 450/80 nm
Flow cell	5-µl volume, 6-mm path length
Slit width	4 nm

Figure 3
Area and retention-time reproducibility of an HPLC peptide standard sample (10 consecutive runs)

Of equal importance is good resolution of solvent composition. For gradients starting at 1 % solvent B at a flow rate of 50 µl/min the high-pressure gradient pumping system must precisely deliver 0.5 µl/min from channel B. The delivery of this flow rate with 1 % composition resolution requires a flow control resolution of 5 nl.

As described above a small delay volume is a must for non-distorted gradient profiles. For the analyses with the 1-mm id column shown in figure 2 the optional precolumn static mixer (420-µl volume) was removed and the autosampler was programmed to bypass the sample loop (about 300-µl volume) automatically after injection. Thus the total delay volume was about 350 µl.

The column temperature stability is another dominating factor because retention times are shortened at elevated temperature. A thermostatted column compartment with good ambient temperature rejection ensures stable chromatographic conditions that are not influenced by periodic fluctuations in room temperature during 24-hour use (see figure 4).

To maintain column performance, short 0.12-mm connecting capillaries and a 1.7-µl volume, 6-mm path length flow cell were used.

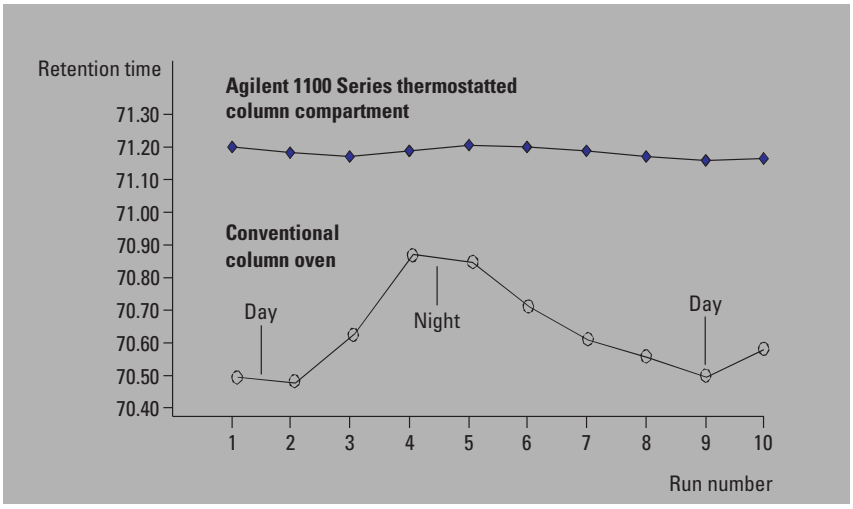
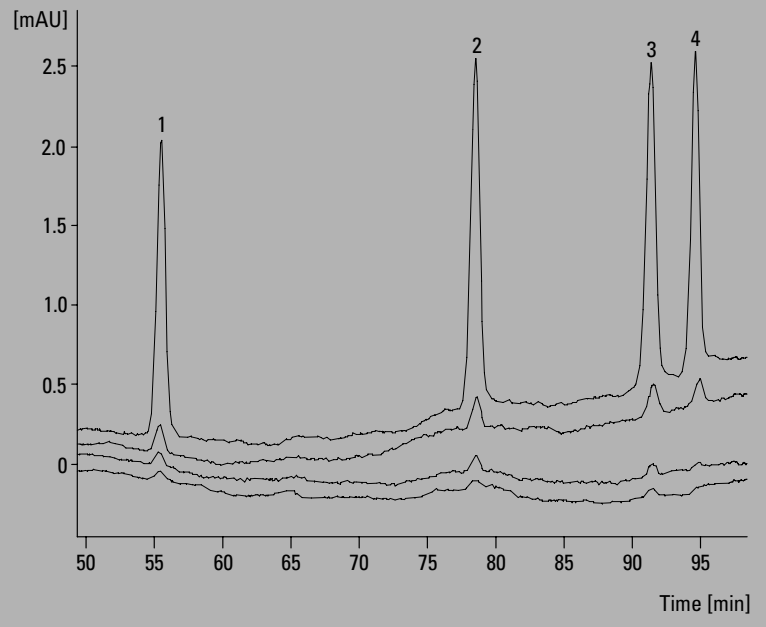


Figure 4
Retention-time fluctuations of a peptide peak over a sequence of 10 consecutive tryptic peptide maps

The highly-reproducible peak areas shown in figure 3 demonstrate the usefulness of an autosampler with excellent precision for small injection volumes, even from sample volumes of only 5 µl.

Subpicomole detectability

The use of 1-mm id columns in bioscience analyses has rapidly increased because of sensitivity advantages in sample-limited applications. In addition, lower solvent consumption and thus lower disposal costs have become important aspects. However, when the focus is on better detectability of low sample amounts, not only the choice of the column affects the results—the instrumental characteristics are also critical in the generation of high-sensitivity peptide maps (see figure 5).



S/N* / Peak	1	2	3	4
5 ng/µl	120.0	146.7	133.3	133.3
0.5 ng/µl	10.6	13.3	13.3	10.7
0.25 ng/µl	4.6	6.7	4.7	2.7
0.125 ng/µl	3.3	2.7	2.7	n.d.

* Calculated with 0.015 mAU noise

Column	1 × 250 mm filled with Vydac TP218
Solvent A	0.05 % TFA in water
Solvent B	0.043 % TFA in acetonitrile
Static mixer	With mixer
Flow rate	50 µl/min
Temperature	40 °C
Gradient	0 min, 1 %B 200 min, 51 %B 210 min, 70 %B 210.01 min, 70 %B, 0.12 ml/min 215 min, 98 %B, 0.12 ml/min 217 min, 98 %B, 0.12 ml/min 220 min, 1 %B, 0.12 ml/min 240 min, 1 %B, 0.12 ml/min 240.01 min, 1 %B, 0.05 ml/min 250 min, 1 %B
Sample	5, 0.5, 0.25 and 0.125 ng/µl HPLC peptide standards 0.125 ng/µl corresponds to 130–375 fmol of the analytes
Injection	1.1 µl of each standard
Detection	214/8 nm, reference 450/80 nm
Flow cell	1.7-µl volume, 6-mm path length
Slit width	4 nm

Figure 5
Detectability of a peptide sample at various concentrations

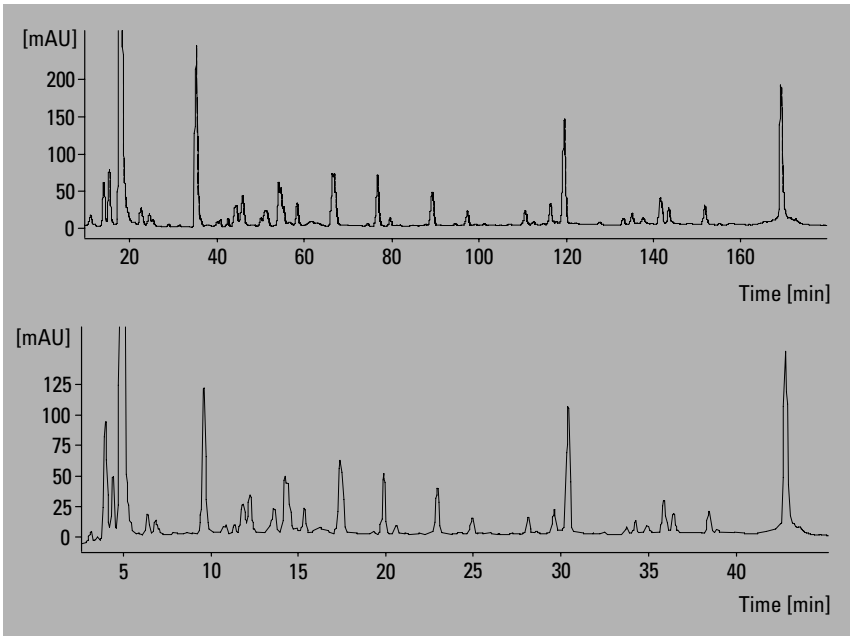


Figure 6
Comparison of two peptide maps at different flow rates but equal volume gradient slope

Column	1 × 250 mm filled with Vydac TP218
Solvent A	0.05 % TFA in water
Solvent B	0.043 % TFA in acetonitrile
Static mixer	Without mixer
Flow rate	Upper map: 50 µl/min Lower map: 200 µl/min
Temperature	40 °C
Gradient (upper map)	0 min, 1 %B 200 min, 51 %B 210 min, 70 %B 210.01 min, 70 %B, 0.12 ml/min 215 min, 98 %B, 0.12 ml/min 217 min, 98 %B, 0.12 ml/min 220 min, 1 %B, 0.12 ml/min 240 min, 1 %B, 0.12 ml/min 240.01 min, 1 %B, 0.05 ml/min 250 min,1 %B
Gradient (lower map)	0 min, 1 %B 50 min, 51 %B 52.5 min, 70 %B 53.75 min, 98 %B 54.25 min, 98 %B 55 min, 1 %B 63 min,1 %B
Sample	Tryptic digest of myoglobin 3.3 mg/ml
Injection	1.1 µl, 190 pmol
Detection	214/8 nm, reference 450/80 nm
Flow cell	1.7-µl volume, 6-mm path length
Slit width	4 nm

As before the solvent delivery system is of importance because low solvent mixing noise provides smooth baselines and enables excellent detectability. For the data shown in figure 5 the high-pressure gradient pump was equipped with a static mixer and the compressibility parameters were optimized for the lowest pressure ripple. This reduced the pumping noise to a level similar to or below the detector noise—even with solvent systems comprising water and acetonitrile, where the composition pulsation is amplified by the presence of TFA.

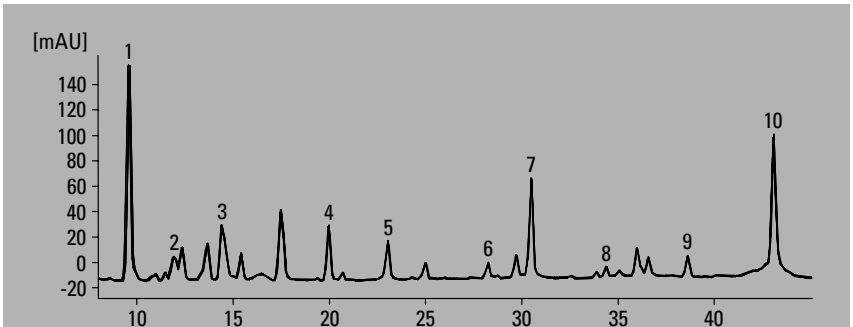
The predominant influence was most certainly the detector. Only low-noise UV detectors ($\pm 1 \times 10^{-5}$ AU, see figure 5), preferably diode-array detectors, provide adequate sensitivity for subpicomole analyses of peptides. To reduce baseline drifts during the gradient the design of the diode-array detector was optimized for low refractive index influences. By setting a reference wavelength close to the measurement wavelength, baseline drifts were reduced significantly.

Shortening analysis times through high-speed peptide mapping

Peptide mapping on 1-mm id columns packed with 5-µm, 300-Å material is usually performed at a flow rate of 50 µl/min (with a back pressure of about 40 bar). The total analysis times are often 2–3 hours as a result of the shallow gradients that are used.

If the back pressure during the analysis does not exceed 350 bar, such colums can also be operated at higher flow rates. As long as the gradient slope—measured in volume units—is kept constant, the flow rate will have little effect on the separation efficiency. For columns packed with particles ≤ 5 µm the slope of the C-term in the van Deemter curve, which describes the loss in plates with increasing flow rate, is small. The actual loss in plates when increasing flow rate is therefore relatively small. In addition, because resolution is proportional only to the square root of the plates, little change in resolution can be expected.

However, the parameter *resolution at different flow rates* is often estimated by changing the flow rate without changing the gradient time-program. Under



Peak No.	1	2	3	4	5	6	7	8	9	10
Mean RT	9.60	11.90	14.41	19.97	23.05	28.25	30.51	34.39	38.62	43.07
SD RT	0.041	0.027	0.020	0.021	0.018	0.018	0.014	0.020	0.019	0.022
RSD RT	0.43	0.22	0.14	0.10	0.08	0.07	0.05	0.06	0.05	0.05

Figure 7
Retention-time reproducibility over eight runs of a high-speed peptide map (conditions as in lower peptide map of figure 6)

these conditions significant differences in selectivity are found which are caused by the different gradient slope (% B/ml) and not by the flow-rate change. As pointed out before, the composition change/volume must be kept constant to see the impact of flow rate on resolution. For example, when the flow rate is doubled the gradient slope in the time program must also be doubled to maintain the same slope in volume units.

In Figure 6 a traditional peptide map (50 µl/min with 0.25 % B/min) is compared to a map produced at a flow rate of 200 µl/min with 1 % B/min. By increasing the flow rate as well as the gradient steepness by a factor of four, analysis time could be decreased by the same factor—without significant decrease in resolution. The results shown here are therefore in good agreement with the theory. The rapid peptide separations on 1-mm id columns also showed excellent retention-time reproducibilities (see figure 7).

Because the peak volume remains unchanged this method exhibits no drawbacks for further microanalytical steps where low volumes with high peptide concentrations are needed.

Conclusion

For peptide mapping on microbore columns total LC system optimization is needed because the performance of each module influences result quality. In Agilent 1100 Series systems instrumental prerequisites for reliable peptide mapping have been carefully optimized, which has been demonstrated by excellent reproducibility and detectability data.

Although microbore peptide mapping is traditionally performed at a flow rate of 50 µl/min, it can easily be performed at 200 µl/min, decreasing the analysis time by a factor of four without significant loss in resolution.

Abbreviations

HPLC High-performance liquid chromatography
TFA Trifluoroacetic acid

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