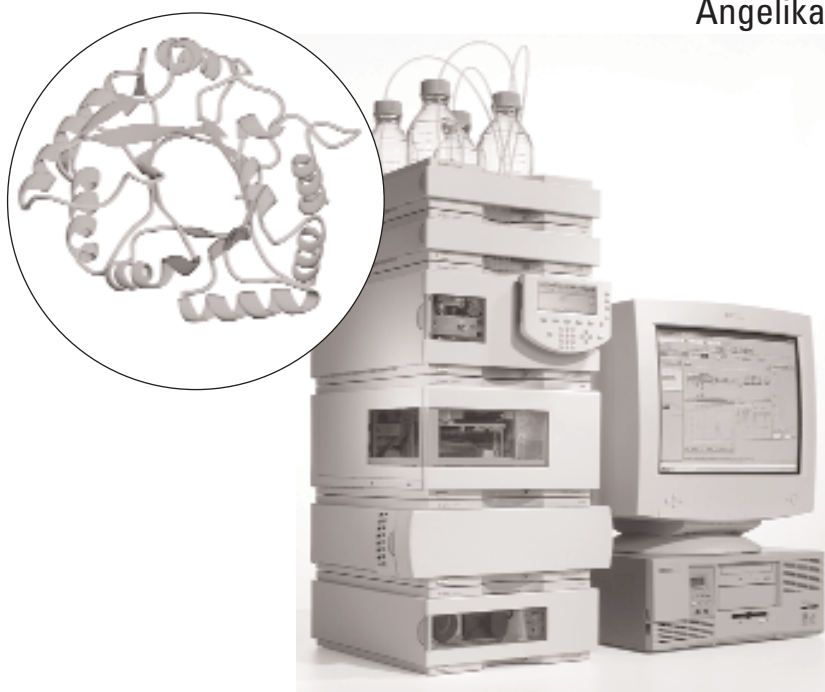# Peptide and protein analysis by capillary HPLC – Optimization of chromatographic and instrument parameters

Application

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This application has been verified using an Agilent 1200 Series LC system, and showed comparable or even better performance.

Application Area
Peptide and protein analysis

Abstract

The analysis of biologically active peptides, peptides from digests and proteins is influenced by many parameters – typical chromatographic parameters, for example, column and gradient, but also instrumental parameters, such as pump, injector or detector setup. The Agilent 1100 capillary system is designed such that it can be treated and set up like a standard LC instrument. Having optimized all instrumental and chromatographic parameters, the relative standard deviation for retention times for peptides and proteins can be expected to be smaller than 0.5 %. This also allows to use retention times in long shallow gradients for confirmation of peak identity, complementary to MS detection.

Agilent Technologies

Introduction

Capillary reversed phase HPLC has become an indispensable tool for analysis of peptides, proteins and digests. Columns with small internal diameter provide improved sensitivity when sample volume and/or sample concentration is very low. In 1999 Agilent Technologies introduced the Agilent 1100 Series capillary LC system, which fulfills these requirements, offering excellent performance and robustness.

The Agilent 1100 Series capillary pump is based on the Agilent 1100 Series high-pressure binary pump. Flow rates down to 1 µl/min are delivered using an electronic flow control (EFC) device, which consists of an electromagnetic proportioning valve and an electronic flow sensor. The column flow is actively measured and kept stable even at changing pressures.[4]

In this Application Note we demonstrate how chromatographic and instrument parameters can be optimized for best performance when analyzing peptides, proteins and digests. The influence of the following parameters is discussed:

Chromatographic parameters
• column
• mobile phase
• gradient
• column temperature
• different pH
Instrument parameters
• injector
• detector
• pump
• column compartment

Equipment

For the experiments the Agilent 1100 capillary LC system used consisted of:

• Micro vacuum degasser with an internal volume of 1 ml for fast solvent changeover
• High-pressure capillary pump for stable flow rates in the low µl range
• Micro well-plate autosampler for precise injections in the nl- and µl-ranges with sample cooling device to avoid sample decomposition
• Thermostatted column compartment for stable retention times at any temperature between 10 °C degrees below ambient and 80 ºC.
• Diode array detector with a 500 nl cell for sensitive analysis of small peak elution volumes

The samples used were purchased from Agilent, Sigma and Michrom:
- Sigma HPLC Peptide standard H-2016
- Sigma HPLC Protein Standard H-2899
- Sigma Lysozyme L-6876
- Sigma Cytochrom C-7752
- Sigma Myoglobin M-1882
- Sigma Ribonuclease R-5503
- Sigma Insulin I-5500
- Sigma Met-Lys-Bradykinin B-5014
- Sigma Leu-Enkephalin E-8757
- Sigma Met-Enkephalin M-6638
- Sigma Angiotensin II A-9525
- Agilent Angiotensin I G 1982-85000
- Michrom tryptic digest of Apotransferrin 910/000/12/26

The amino acid sequence of the used peptide and the formula and molecular weight ranges of peptide and proteins are listed in table 1.

Amino acid sequence	Formula and molecular weight ranges
Met-Enkephalin:	Try-Gly-Gly-Phe-Met
Leu-Enkephalin:	Try-Gly-Gly-Phe-Leu
Leu-Enkephalin-Arg:	Tyr-Gly-Gly-Phe-Leu-Arg
Angiotensin 1:	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu
Angiotensin 2:	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe
Met-Lys-Bradikinin:	Met-Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg
Gly-Tyr and Val-Tyr-Val:	FW 239- 381
Met-Enkephalin, Leu-Enkephanlin and Leu-Enkephalin-Arg:	FW 557-713
Met-Lys-Bradikinin, Angiotensin 1and 2:	FW 1046-1320
Apo-myoglobin:	MW ~ 17800
Cytochrom c and Ribonuclease a:	MW ~12173- 13700
Holo-transferrin:	MW ~76000 - 81000

Table 1
Amino acid sequence and formula and molecular weight ranges of peptides and proteins

The following capillary columns were used:
1. Zorbax 150 x 0.3 mm 300Å Extend C-18, 3.5 µm, (Agilent part number 5065-4464). These columns incorporates bidentate silane, combined with a double-endcapping process that protects the silica from dissolution at high pH.
2. Zorbax 150 x 0.3 mm 80Å XDB C-18, 5 µm (Agilent part number

5064-8291). The densely-bonded, double endcapped silica surface minimizes any silanol interactions.

3. Zorbax 150 x 0.3 mm 80Å SB C18, 5 µm (Agilent part number 5064-8255). SB columns are made using bulky unique silanes that sterically protect the siloxane bond. Stable bond material is not endcapped.

4. Zorbax 150 x 0.3 mm 300Å SB C18, 3,5 µm (Agilent part number 5064-8267).

5. Zorbax 150 x 0.3 mm 300 SB C-8, 3.5 µm (Agilent part number 5065-4460).

6. Zorbax 150 x 0.3 mm 80Å Eclipse XDB C-18, 5 µm, (Agilent part number 5064-8291). The densely-bonded, double-end capped silica surface minimizes any silanol interaction and protects the silica support from dissolution.

7. Hypersil 120Å ODS C-18 150 x 0.3 mm, 5 µm (Agilent part number 5064-8290). A very polar packing, adds a monolayer of octadecyl silane (C-18) to the Hypersil support. Columns are endcapped to reduced silanol interaction.

For more column information refer to literature reference 1, which is a reference guide for columns and supplies[1]. This guide is updated on a yearly base and contains detailed information about different column properties.

Results and discussion

Influence of different column materials
Reversed phase columns are frequently used for the separation of peptides, proteins and tryptic digests, since reversed phase provides optimum resolution. It has been demonstrated that reversed-phase HPLC provides separation of polypeptides of nearly identical sequences.[2] The separation mechanism of peptides and proteins on reversed phase column is based on small differences in the "hydrophobic foot" of the polypeptide, based on differences in amino acid sequences and differences in confirmation.[2] Whereas the separation mechanism of small peptide on reversed phase is assumed to be a mixture of

adsorption and partition, the separation of big proteins is based on adsorption only.[3] The elution of these big molecules depends mainly on the organic phase concentration. If for one of these molecules, which is adsorbed at the surface of the column material, the critical percentage of organic phase is reached the molecule leaves the surface and is transferred to the detector without further interference with the stationary phase.

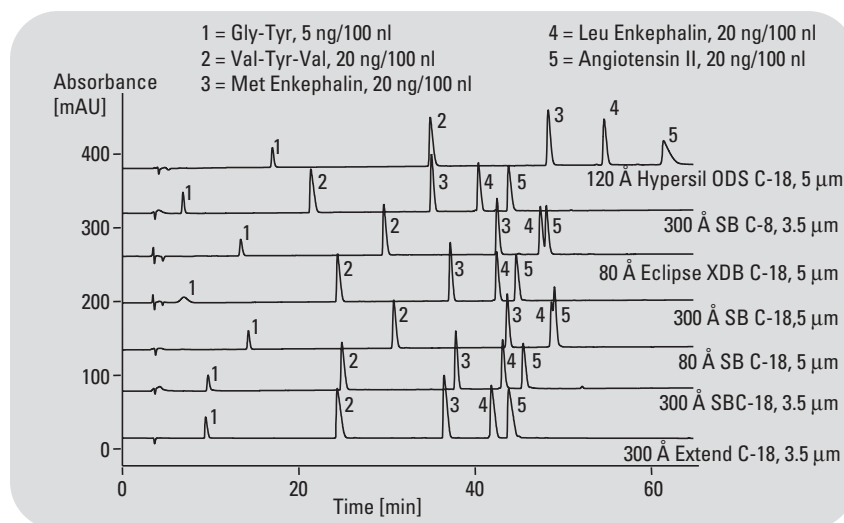To demonstrate the influence of different column material on the

Figure 1
Analysis of a peptide standard on different capillary columns of the same length and internal diameter.

Chromatographic conditions

Column flow:	5.5 µl/min
Primary flow:	low solvent consumption: 200-500 µl/min
	(Optimum pump settings will be explained later.)
Mobile phases:	water + 0.05 % TFA, pH = 2.2 = A, acetronitrile + 0.045 % TFA = B
Gradient 0.5 % B/min:	at 0 min = 1 % B, at 60 min = 31 %B, at 70 min = 50 % B,
	at 75 min = 85 % B, at 80 min = 85 % B, at 81 min = 1 % B, at 110 min = 1 % B
DAD UV detector:	206/10 nm, ref 450/80 nm
Injection volume:	0.1 µl, automatic delay volume reduction was activated(will be explained later)
Column temperature:	30 °C

separation of polypeptides seven different capillary columns were tested. All tested capillary columns are available from Agilent Technologies. The following different column material was tested:

- Different C-18 materials, silica material end-capped vs. non end-capped, polar vs. non-polar
- Different pore size, 80Å vs. 300Å
- Different polarity of reversed bonded phases, C-18 vs C-8

Figure 1 shows an overlay of chromatograms of a peptide standard, analyzed on seven different reversed phase columns. The Hypersil ODS material, which is a polar packing, gives long retention times and tailing peak shape. The last peak, which is Angiotensin II with a formular weight of 1046.2 already shows a significant tailing. All other tested columns with less polar material provide better peak shapes. The Zorbax 300Å Extend material and all other 300Å show shorter retention times and good peak shape. All tested 300Å materials are able to separate the injected compounds under the conditions used. The 80Å materials also show good peak shape and short retention times but are not able to completely separate compounds 4 and 5. The 300Å C-8 material is also able to separate all peptides and together with the 300Å Extend C-18 material offers the shortest retention times. In figure 2 another peptide standard was analyzed using the same columns and the same chromatographic parameters as in figure 1. The polar ODS Hypersil column also shows long retention times and peak tailing. The 300Å C-8 and
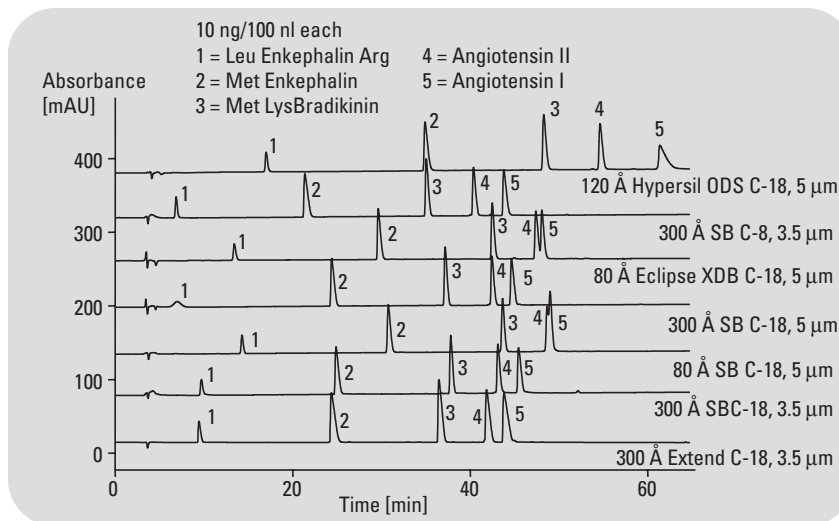

Figure 2
Analysis of another peptide standard using the same columns and chromatographic conditions as in figure 1.
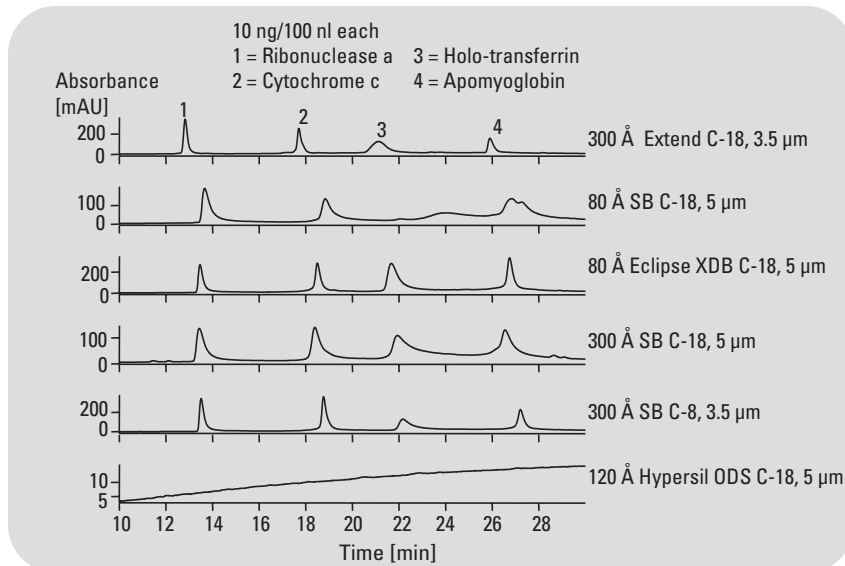

Figure 3
Analysis of proteins on six different capillary columns with a length of 150 mm and an internal diameter of 0.3 mm.

Chromatographic conditions

Column flow:	5.5 µl/min
Primary flow:	low solvent consumption
Mobile phases:	water + 0.05 % TFA = A, acetonitrile + 0.045 % TFA = B
Gradient:	At 0 min = 20 % B, at 60 min = 85 % B, at 61 min = 90 % B, at 65 min = 90 % B, at 66 min = 20 % B, at 80 min = 20 % B
DAD UV detector:	206/10 nm, ref 450/80 nm
Injection volume:	100 nl, automatic delay volume reduction activated
Column temperature:	30°C

the 300Å Extend C-18 offer the shortest retention times. Here only the 80Å C-18 columns are able to separate all compounds to baseline. Obviously it is not possible to recommend just one column for the separation of peptides with MW < 2000. Further experiments are still necessary to find the optimum column for a specific application. In the beginning of method development it makes sense to test three or four columns of the same length, using the same chromatographic conditions, and to compare the separation efficiency on:

- Non-polar packing materials with pore size of 300 vs. 80Å
- C-18 vs C-8 bonded phase material or others bonded phases like phenyl or C-4

This should help to find the optimum column for a specific application.

The next experiments show the separation of proteins with molecular weights > 10000. Again, prediction based on molecular weight is not possible. Holo-tranaferrin, which is by far the biggest molecule is not the last one in the chromatogram, as may be expected. Figure 3 shows the chromatograms of a protein standard, analyzed on six different columns. It is quite obvious that the polar Hypersil column is not suited for this type of application. Very good peak shape could be achieved on the 300 Extend C-18 column. This column is specifically designed for reversed-phase separation of peptides, proteins and the analysis of peptide fragments from enzymatic digests.
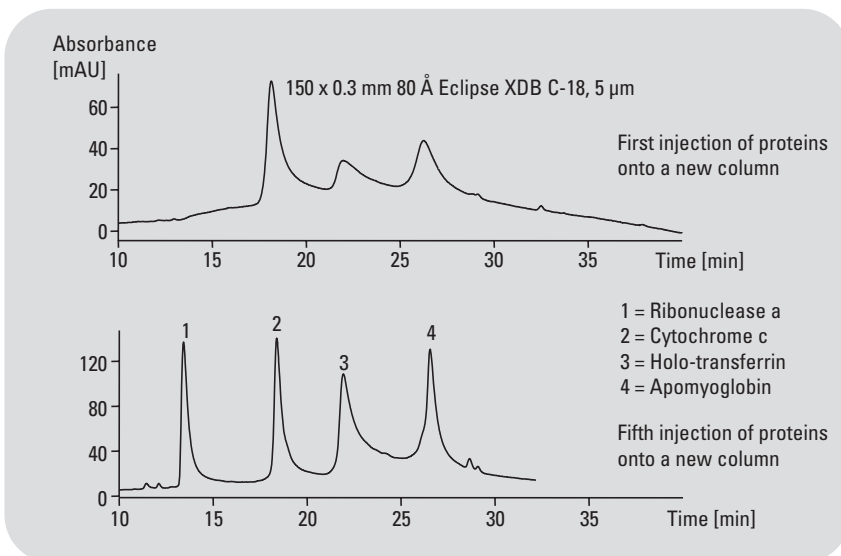

Figure 4
Change of chromatogram from the first protein injection to the fifth injection of proteins, chromatographic conditions as figure 3, column 150 x 0.3 mm 80Å Eclipse XDB C-18, 5 µm.

For protein analysis it is important that the column used has "seen" proteins before. This means that several injections of a protein standard should be done onto a new column. If after several injections the chromatograms all look the same and retention time precision is at least below 0.5 % RSD, calibration and sample runs can be started.

Figure 4 is an example of how different chromatograms appear on a new column and after several protein runs. Not all columns show such a significant difference. Columns, which are specially designed for protein analysis, show less difference for the first and later runs. Having selected the column, other parameters such mobile phase, gradient, concentration of modifier detection wavelength, pH and column temperature have to be optimized. For all

further experiments we will use the column 150 x 0.3 mm 300Å Extend C-18, 3.5 µm.

Influence of mobile phase fluctuations on retention times
For all chromatograms above water and acetonitrile with trifluoracetic acid (TFA) as modifier were used. TFA is the preferred ion-pair reagent for best peak shape. Another advantage is that TFA absorbs in the UV range below 200 nm and therefore shows minimal interference with detection of peptides above 200 nm. One disadvantage of TFA is that it causes baseline drift due to spectral absorption differences with changing acetonitrile percentage. To reduce this drift as much as possible the TFA concentration in organic phase, here acetonitrile, should be 15 % lower than in the water phase.

Unfortunately, TFA is not the best choice for mass spectrometers, because it suppresses the ion generation. Ion suppression can be partially overcome by lowering the TFA concentration in the mobile phase. For some columns lowering the TFA concentration might lead to broader and tailing peaks, however the 300Extend C-18 column does not have such a problem, as shown in figure 5. In this case lowering the TFA concentration resulted in the separation of the first two peaks, which were not separated using the higher TFA concentration.

In figure 6 an example is given on how lower TFA concentration influences the separation of peptides from a myoglobin digest. No additional peak tailing can be observed and separation efficiency is also not negatively effected. Nevertheless, in MS applications it has become good practice to use formic acid (FA) as modifier instead of TFA. Formic acid is also volatile and does not suppress ion generation like TFA.
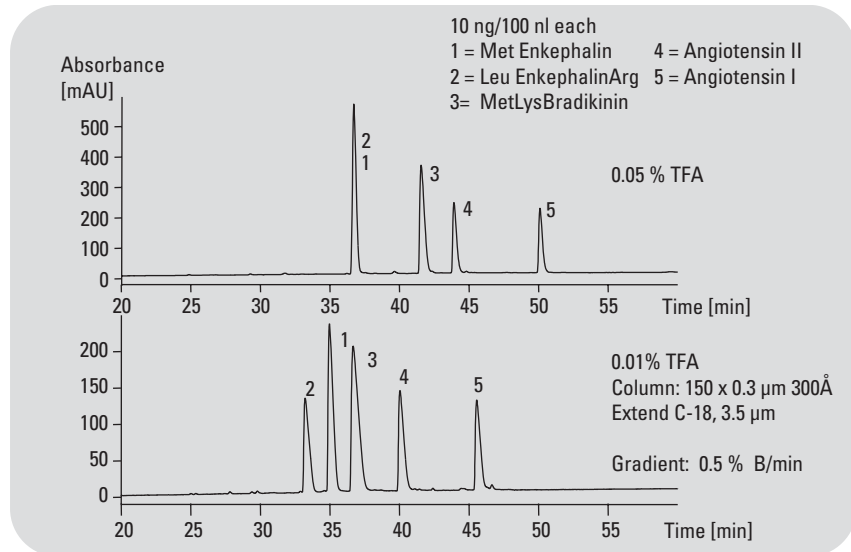

Figure 5
Analysis of peptides at different TFA concentrations, all other conditions are the same as in figure 1.
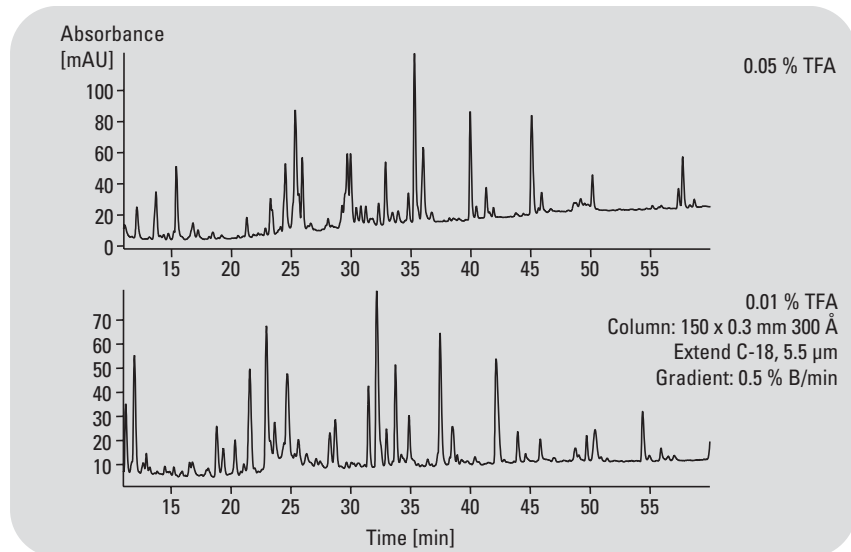

Figure 6
Analysis of myoglobin digest using different TFA concentrations. All other conditions are the same as in figure 1, except that the injection volume was 3 µl, which represents an injection of 3 pmol.

Figures 7, 8 and 9 show the influence of formic acid on peak shape and elution series of a peptide standard, a digest and a protein standard. Analyzing peptides and proteins with formic acid instead of TFA may lead to broader peaks and different elution times for the injected compounds. In our example the elution order has changed. Met-Lys-Bradikinin has moved to lower retention time and now elutes between Leu-Enkephalin-Arg and Met-Enkephalin. This is due to the fact that Bradikinin has 2 Arg in its sequence which both form ion pairs with TFA. This results in longer retention times for Bradikinin, if TFA is used as mobile phase modifier.

Using formic acid as mobile phase modifier for the analysis of peptides shows no or only minor disadvantages for peak shape. For big proteins as seen in figure 7 formic acid is not ideal.
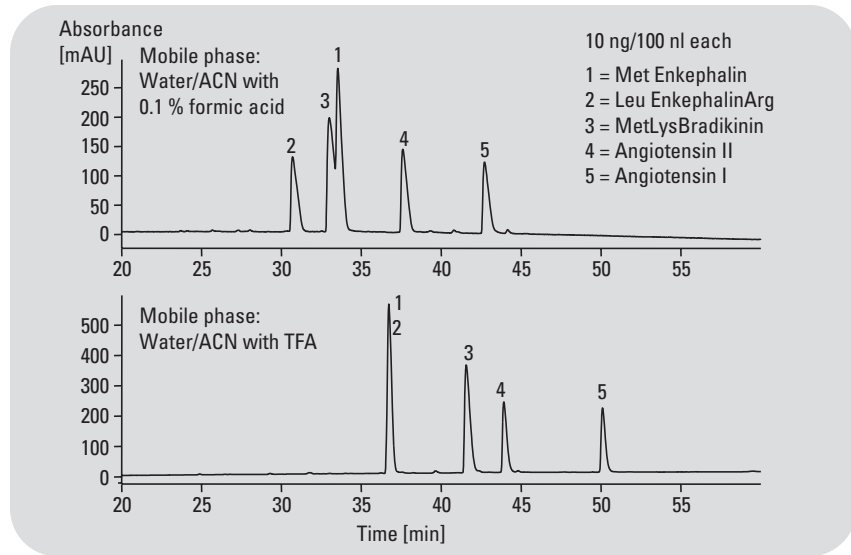

Figure 7
Analysis of a peptide standard using different mobile phases, all other conditions are the same as in figure 1.
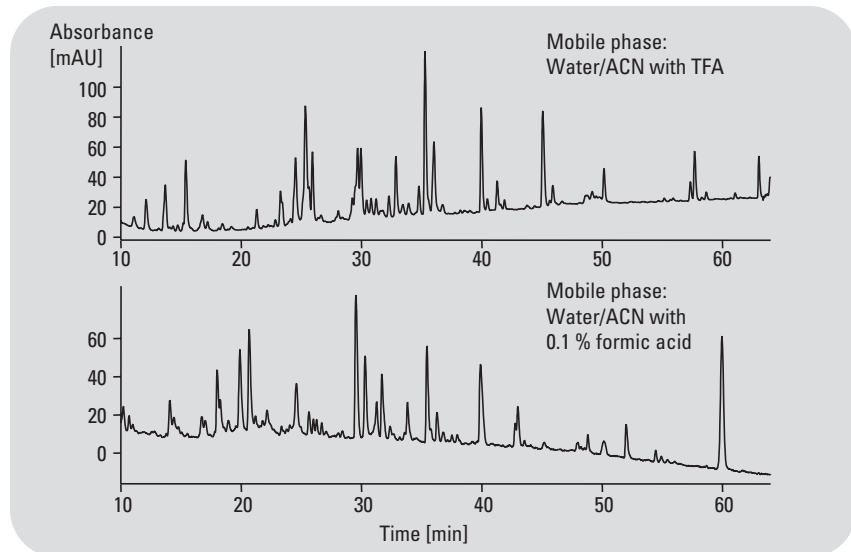

Figure 8
Analysis of a peptides from a Myoglobin digest standard using different mobile phases. All other conditions are the same as in figure 1, except that the injection volume was 3 µl, which represents an injection of 3 pmol.

Influence of gradient settings

As already mentioned small changes for chromatographic parameters can influence peptide or protein separation significantly. This is definitely true if gradient settings are changed. In figure 10 two different gradients were applied to the same sample. The shallower gradient gives at least a partial separation of the first two peaks. Using shallow gradient also prolongs retention times significantly. For specific peptide separations it is of advantage to test more rapid gradients first. This can help to save cycle times, labor time and costs.

Using shallow gradients for peptide maps offers a much better separation performance for the analysis of peptides from a Apotransferrin digest (figure 11). Shallow gradients for peptide maps are recommended, if optimum separation for identification should be obtained.

Typically, shallow gradients provide better resolution for peak clusters. In the example above the peak cluster at approximately 20 and 40 minutes show better resolution with 0.25 % organic phase increase per minute.
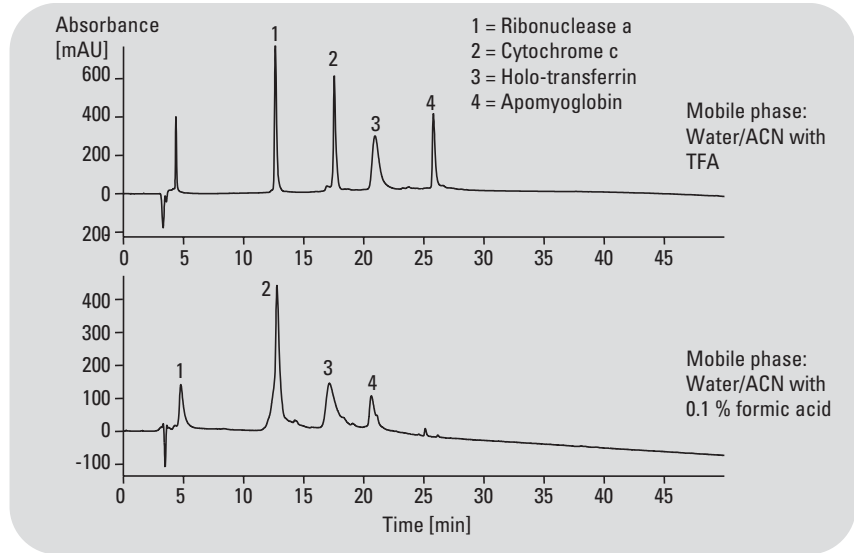
Figure 9
Analysis of a peptides from a protein standard using different mobile phases, all other conditions are the same as in figure 3.
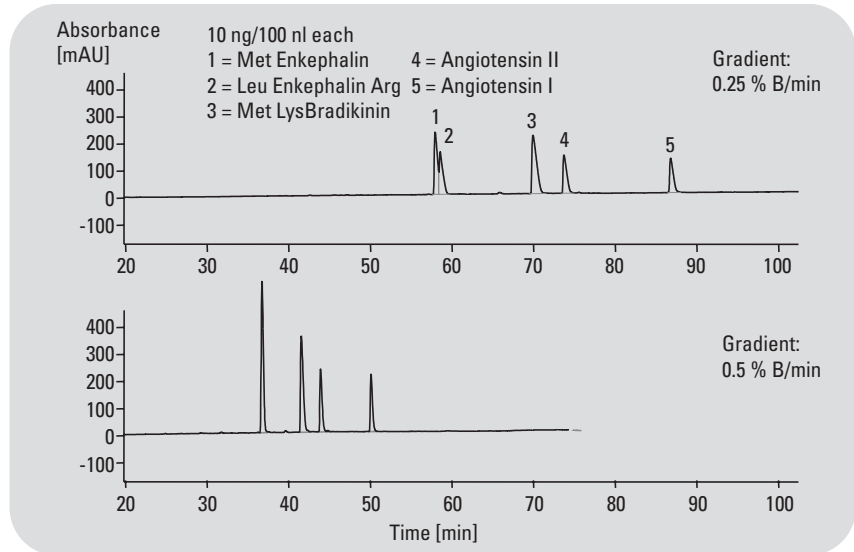
Figure 10
Analysis of peptide standard using different gradients. All other conditions are the same as in figure 1 except the gradient. The gradient was changed to reach 31 % B in 120 min to provide an increase for the gradient of 0.25 %/min.

Influence of column temperature

Different column temperatures can also be an important tool to improve separation efficiency. In figure 12 the influence of the column temperature on the separation of a peptide standard is shown. In this example increasing the column temperature provides better separation of the first two peaks. A further effect is that the run time could be shortened by 10 minutes.
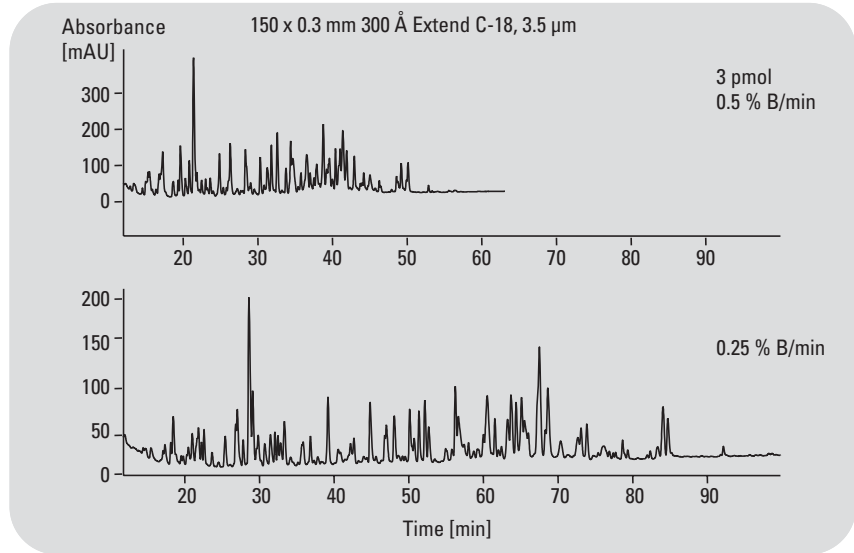

Figure 11
Analysis of Apotransferrin digest using different gradients. All other conditions are the same as in figure 1 except the gradient. The gradient was changed to reach 31 % B in 120 min to provide an increase for the gradient of 0.25 %/min. Injection volume was 3 µl.
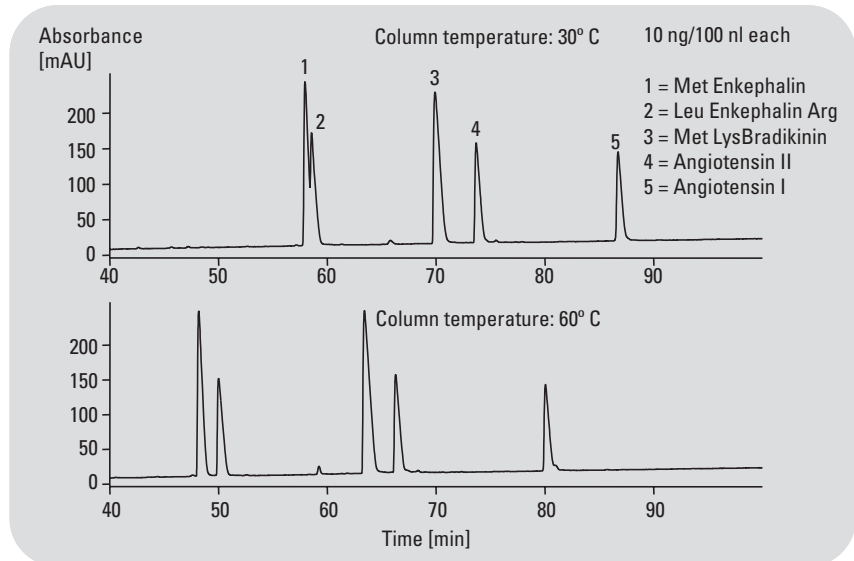

Figure 12
Analysis of peptide standard at different column temperatures, all other conditions are the same as in figure 1.

Influence of different pH

The separation of peptide is also strongly influenced by the pH, due to protonation and deprotonation processes of basic or acidic side chains. Figure 13 shows how strong the influence on the elution of peptides can be. A peptide standard was analyzed using 0.001 m NaH_2PO_4 with pH 7 and 0.05 % TFA modifier with pH 2.2 as mobile phases. The elution series is completely different for Met-Enkephalin, Leu-Enkephalin and Angiotensin II, if the pH changes so drastically. The smaller peptides are less effected in this case. It is also obvious that with the higher pH the retention times shift to shorter value.
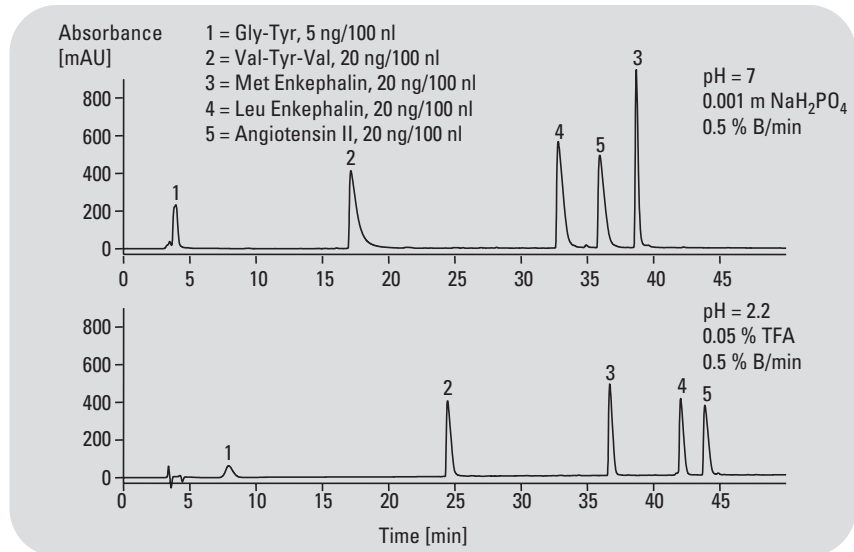
Detector settings for the Agilent 1100 Series diode array detector

For combination with low flow rates the 1100 Series diode array detector (DAD) is equipped with a 500-nl cell, which provides lowest peak dispersion in combination with capillary columns with ids down to 300 mm. The 1100 Series DAD offers the possibility to acquire several UV signal simultaneously. Figure 14 shows how different UV signals can provide additional information.

In addition to the absorption around 206 nm cytochrome has a maximum at 400 nm. Proteins containing Trypthophan or/and Tyrosine also show some absorption at 280 nm. In combination with for example MS data, this can help to identify peptides or proteins more easily.
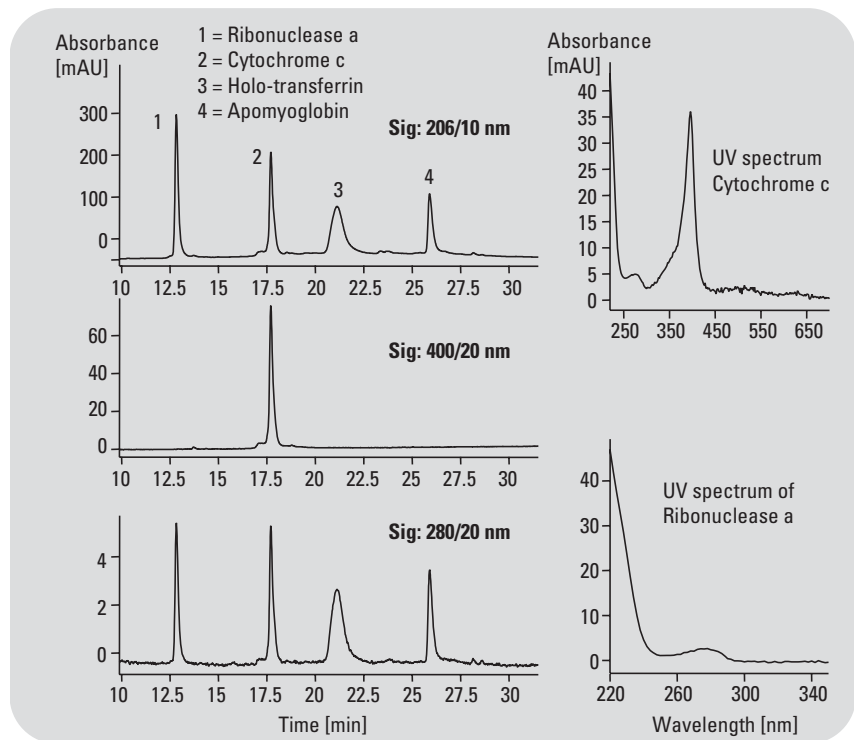

Figure 13
Analysis of a peptide standard at different pH, all other conditions are the same as in figure 1.

Figure 14
Analysis of a protein standard at different wavelength, all other conditions are the same as in figure 3.

Injector settings for the Agilent 1100 capillary instrument

In its standard version the Agilent 1100 Series micro-well plate sampler is equipped such to allow injection volumes from 0.01 up to 8 µl. Changing the loop capillary extends the injection volume to 40 µl. Typically this gives sufficient flexibility in the analysis of peptides, proteins and digests. The area precision for different injection volumes is given in table 2. For more information refer to literature reference 4.

The lower the flow rates the more important the system delay volume gets. For a capillary system small delay volumes between point of injection and column head and column end and detector cell are of utmost importance for low peak dispersion. Also, the detector cell should have a low volume. The delay volumes for the 1100 Series capillary system are 0.08 µl before and after the column. The internal volume of the detector is 500 nl. For lowest delay volume it is recommended to activate the "automatic delay volume reduction" function in the setup screen for the micro well-plate sampler. This allows to bypass the autosampler delay volume after the sample has reached the top of the column. This saves 300 µl delay volume of the autosampler and is the main step to reduce the system delay volume. The delay time is about 50 % lower.

Injection volume of Met-Enkephalin (ml) (concentration = ~1ng/µl)	RSD retention times in % (n=6 each)	RSD areas in % (n=6 each)
0.03	0.14	7.59
0.1	0.19	2.69
0.5	0.06	1.91

Table 2
Precision of areas at different injection volumes

Pump settings

The column flow is set according to the internal diameter of the column. The primary flow rate, which determines the flow rate delivered by the pump up to the electromagnetic proportioning valve, can be selected within three ranges.

1. Low solvent consumption, 200 to 500 µl/min
2. Medium solvent consumption, 500 to 800 µl/min
3. High solvent consumption, 800 to 1300 µl/min for rapid gradient changes

In the electromagnetic proportioning valve the flow is split into the column flow and the "waste"flow. The range selected for the primary flow depends on the application. For typical peptide and protein analysis the first range is selected. If speed of analysis is an important issue range 2 or 3 can be selected.

Other important pump settings, which influence pump performance, are compressibility and the calibration settings for the flow sensor. Because the flow measuring process is based on heat capacities, each solvent mix needs its individual calibration curve, which is available from the ChemStation or control module for most common solvents and solvents mixtures. Also, for the compressibility parameter the ChemStation proposes values for the most common solvents and solvent mixtures.

Having entered all settings according to the used solvents, the precision of retention times is < 0.3 % relative standard deviation (figure 15).

Column compartment set up

It is recommended to connect the flow connection capillary from the injection valve directly to the capillary column and not to the mobile phase pre-heating device. This saves run and cycle time and the compounds do not have contact with metal surface of this device. The capillary column should be installed with column holder to provide good contact with heat zone.

Conclusion

The analysis of peptides, peptides from digests and proteins is influenced by many parameters. These include typical chromatographic parameters, such as column and gradient, but also instrumental parameters, for example, set up of pump, injector or detector. In principle there is no significant difference to standard, narrow-bore or micro-bore HPLC, except that the dimensions are different.

The Agilent 1100 Series capillary LC system is designed such that it can nearly be treated and set up like a standard LC instrument. Only a few settings should be selected with care and the recommendations given by the help function should be followed.
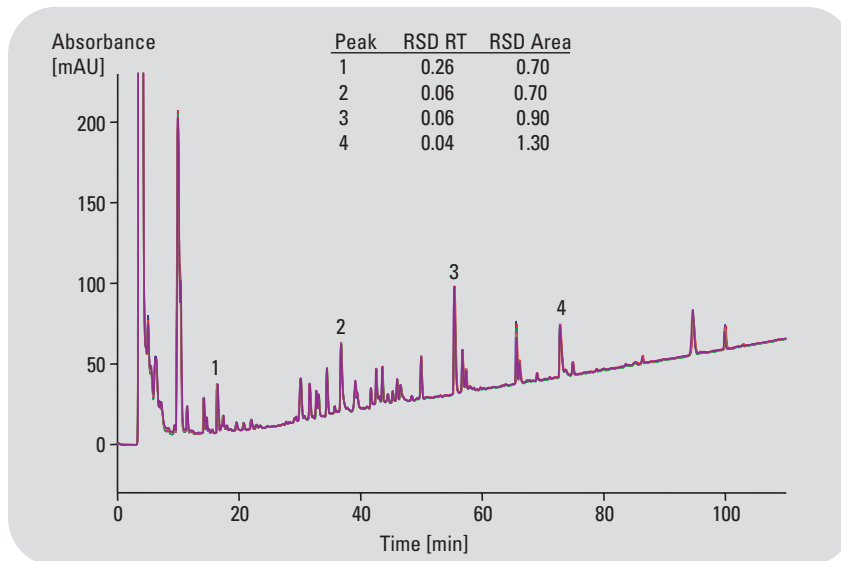

Peak	RSD RT	RSD Area
1	0.26	0.70
2	0.06	0.70
3	0.06	0.90
4	0.04	1.30

Figure 15
Precision of retention times for peptide of a myoglobin digest. All other conditions are the same as in figure 1 except the injection volume with 3 µl.

Recommendations for the chromatographic parameters are somewhat more complex.
• A good start is to select a capillary column which is specially designed for the separation of peptides and proteins, such as the Agilent 300 Extend C-18 capillary column which is available in different lengths.
• The next step is to decide which mobile phase is appropriate for the detector. If a mass spectrometer is used a good choice is to use 0.1 % formic acid as modifier. If a UV detector is used, Trifluoroacetic acid should be used as modifier.

• If the peptides of a digest gradient are be analyzed a shallow gradient is recommended. For the separation of known peptides or proteins it is of advantage to select a gradient which provides sufficient separation in cycle times as short as possible.
• If the separation cannot be achieved with the first set of parameters it is recommended to change the gradient first, followed by changing the column temperature, mobile phase concentration and/or pH.
• If a separation can still not be achieved a different stationary phase with other pore size and/or differently bonded phase should be selected.

Having optimized all instrumental and chromatographic parameters the relative standard deviation for retention times can be expected typically < 0.5 % for a runtime range from 0 to 100 minutes. The area precision for a digest with an injected concentration of 3 pmol and an injection volume of 3 µl is typically < 2 % RSD. Tables 3, 4 and 5 should help to find optimum separation conditions quickly and conveniently. The optimization procedure is limited to reversed phase applications.

Selection of separation mechanism		
Peptide with MW<2000 DA	**Peptides/proteins with MW>2000DA**	**Membrane proteins**
Reversed phase separation	Reversed phase separation, limit is ~30 – 36 k DA or Ion exchange or Size exclusion	Ion exchange separation or Size exclusion with detergents

Table 3
Recommendation for selecting the best column material

Recommended start conditions for reversed phase separation		
Parameter	**Peptides with MW<2000 DA**	**Peptides/proteins >2000 DA**
Column selection	150 x 0.3 mm Zorbax 80 Å SB-18 or/and 150 x 0.3 mm Zorbax 300 Å Extend C-18	150 x 0.3 mm Zorbax 300 Å Extend C-18
Flow rate	5.5 µl/min	5.5 µl/min
Mobile phase	For UV detectors: water and acetonitrile with 0.05 %TFA as modifier For MS detectors: water and acetonitrile with 0.1 % formic acid as modifier	For UV detectors: water and acetonitrile with TFA as modifier For MS detectors: water and acetonitrile with formic acid as modifier or For MS detectors: water and acetonitrile with 0.01% TFA as modifier
Gradient	1 to 31% ACN with 0.5 %/min ACN increase	20 to 85 % ACN with ~1.1%/min ACN increase

Table 4
Recommended start conditions

Recommended optimization process of start conditions		
Parameter	**Known peptides and proteins**	**Peptide maps with unknown compounds**
Gradient	If the start conditions do not provide baseline separation, use shallow gradients with 0.25 %/min ACN increase.	More shallow gradients with 0.25 %/min ACN increase are recommended.
Oven temperature	Start temperature is typically 40 ºC. For improving separation temperature can be raised to 60 ºC	Typically 40 ºC are used
Modifier concentration	Increase and decrease of modifier concentration can be used to optimize separation.	Typically TFA concentration ≤ 0.1 % is used for UV detection If an MS is used as detector 0.1 % formic acid is a recommended concentration
pH	The pH can be varied from acidic to basic conditions as far as the column can tolerate the change	Acidic conditions are typically used, such as pH=2.2 with 0.05 % TFA

Table 5
Recommended optimization process

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