Separation of Large and Small Peptides by Supercritical Fluid Chromatography and Detection by Mass Spectrometry

Application Note

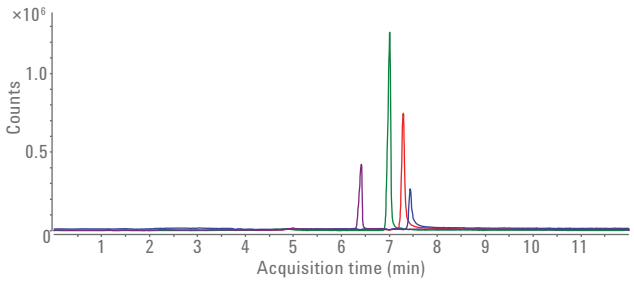
Biologics and Biosimilars

Author

Edgar Naegele
Agilent Technologies, Inc.
Waldbronn, Germany

Abstract

This Application Note demonstrates the use of supercritical fluid chromatography (SFC) for the separation of di- and tri-amino acids and peptides. It shows that the separation of smaller and larger peptides is possible by SFC. For detection, a mass spectrometer was used. For larger peptides occurring in different charge states, Agilent MassHunter Bioconfirm software was used for deconvolution to determine the molecular weight of the peptides.



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Introduction

Today, supercritical fluid chromatography (SFC) could be used in different analytical fields such as the pharmaceutical industry. This is documented by a scientific review written by a large pharmaceutical company[1]. They demonstrated the successful use of SFC/MS for high-throughput SFC/APPI-MS, ultrafast SFC/MS, chiral SFC/MS, and SFC/MS for the analysis of peptides and proteins. The biologically important trans-membrane proteins comprise highly hydrophobic regions with helical structures of amino acids piercing through the cell membrane. They are typically insoluble in water and difficult to analyze by RP-HPLC. Here, SFC has been successfully used to separate highly hydrophobic peptides such as gramicidin and integral membrane proteins such as bacteriorhodopsin[1,2]. The analysis of peptides with various basic and acidic amino acids in their sequence are described up to a 40-mer[3].

This Application Note describes the separation of small peptides and hydrophilic peptides of similar amino acid sequences by SFC, and their detection by mass spectrometry. As an example for the separation of a large peptide, the determination of the molecular weight of the large peptide insulin is demonstrated.

Experimental

Instrumentation

The Agilent 1260 Infinity Analytical SFC System (G4309A) comprised the following modules:

- Agilent 1260 Infinity SFC Control Module
- Agilent 1260 Infinity SFC Binary Pump
- Agilent 1260 Infinity High-Performance Degasser
- Agilent 1260 Infinity SFC Standard Autosampler
- Agilent 1260 Infinity Thermostatted Column Compartment
- Agilent 1260 Infinity DAD with high-pressure SFC flow cell
- Agilent 6460 Triple Quadrupole LC/MS System (G6460C) with Agilent Jet Stream
- Agilent 1260 Infinity Isocratic Pump (G1310B)
- Agilent splitter kit G4309-68715

Instrumental setup

Figure 1 shows the recommended configuration of the Agilent 1260 Infinity Analytical SFC System with the Agilent 6460 Triple Quadrupole LC/MS System.

Column

Princeton Chromatography Inc., 2-Ethylpyridine, 4.6 × 250 mm, 5 µm

Software

- Agilent MassHunter Data Acquisition software for triple quadrupole mass spectrometer, Version 07.01
- Agilent MassHunter Qualitative Software, Version 07.00
- Agilent MassHunter Quantitative Software, Version 07.00
- Agilent MassHunter Bioconfirm Software, Version 07.00
- Skyline Software, McCoss Lab Software, University Washington, Version 3.1, for peptide quantification and targeted proteomics[4]

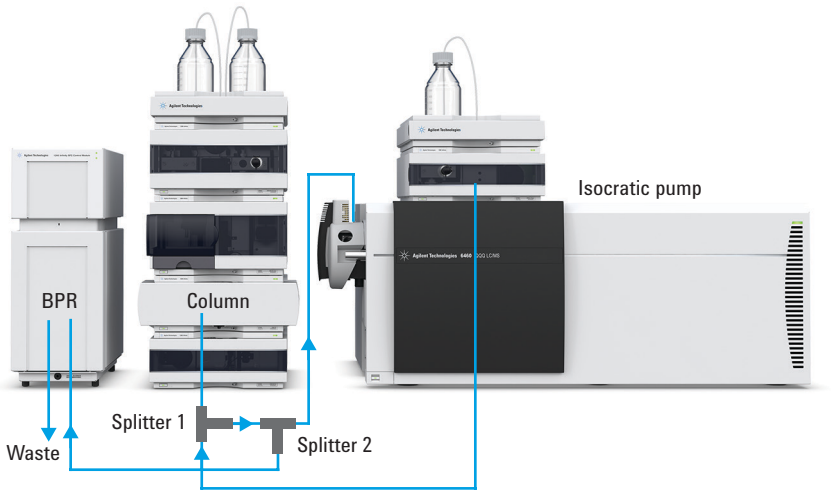

Figure 1. Configuration of the Agilent 1260 Infinity Analytical SFC System with the Agilent 6460 Triple Quadrupole LC/MS System. The column is directly connected to splitter 1 in the splitter assembly (BPR = backpressure regulator, UV detector not used, splitter kit p/n G4309-68715).

SFC method

Parameter	Value
SFC flow	3 mL/min
SFC Gradient 1	0 minutes, 5 %B; 10 minutes, 50 %B
SFC Gradient 2	0 minutes, 25 %B; 10 minutes, 75 %B
Stop time	10 minutes
Post time	2 minutes
Modifier	Methanol + 0.1 % TFA
BPR temperature	60 °C
BPR pressure	150 bar
Column temperature	40 °C
Injection volume	5 µL, three-times loop overfill

Connection of the SFC to the MS by splitting and make-up flow

Parameter	Value
Make-up composition	Methanol/Water (95/5) + 0.1 % TFA
Make-up flow	0.5 mL/min

MS method

Parameter	Value
Ionization mode	positive
Capillary voltage	3,500 V
Nozzle voltage	2,000 V
Gas flow	8 L/min
Gas temperature	220 °C
Sheath gas flow	12 L/min
Sheath gas temperature	380 °C
Nebulizer pressure	25 psi
Fragmentor	130 V
SIM mode used for peptides	See Table 1
MRM mode for Angiotensin II	Transition 1: 532.7 → 784.4, CE: 17 eV Transition 2: 532.7 → 647.3, CE: 17 eV
Scan mode, used for insulin	400–1,600 *m/z*

Table 1. Molecular weight and *m/z* for SIM detection of the analyzed peptides.

Peptide	MW	*m/z*
Gly-Tyr	238.2	239.2
Val-Tyr-Val	379.5	380.5
Leu-enkephalin	555.3	556.3
Met-enkephalin	573.2	574.2
Angiotensin I	1,296.5	433.2
Angiotensin II	1,046.2	524.1
Angiotensin III	931.1	466.5
Angiotensin IV	774.9	388.5

Standard solutions

All peptide samples were prepared at a concentration of 1 mg/mL in make-up solvent, and diluted or mixed to a final concentration of 100 µg/mL.

Chemicals

All chemicals were purchased from Sigma-Aldrich, Taufkirchen, Germany. All solvents were LC/MS grade. Methanol was purchased from J.T. Baker, Germany. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipak).

Results and Discussion

First, the method for the separation of peptides by SFC was developed for di- and tri-amino acids and smaller peptides. As an example, a di- and tri-amino acid were separated by a gradient increasing from 5 to 50 % methanol in 10 minutes on a 2-ethylpyridine column (Figure 2A). Both small peptides were clearly baseline-separated, and eluted in sharp peaks. As examples for smaller peptides, Leu- and Met-enkephalin were used. Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu) and Met-enkephalin (Tyr-Gly-Gly-Phe-Met) are both endogenous opioid peptide neurotransmitters found naturally in the brains of animals, including humans. The tyrosine residue is an analog to the 3-hydroxyl group of morphine with actions on the δ-opioid receptor. Both were separated with the same method that was used for the smaller peptides (Figure 2B). Leu-enkephalin elutes under the chosen conditions at 6.78 minutes, and Met-enkephalin at 7.11 minutes, both with sharp and baseline-separated peaks. All used di- and tri-amino acids, and the enkephalin penta-peptides were detected by SIM-MS as their singly charged ions (Table 1). From repeated injections, the retention time precision and the area precision were determined to be better than 0.05 and 1.75 %, respectively (Table 2).

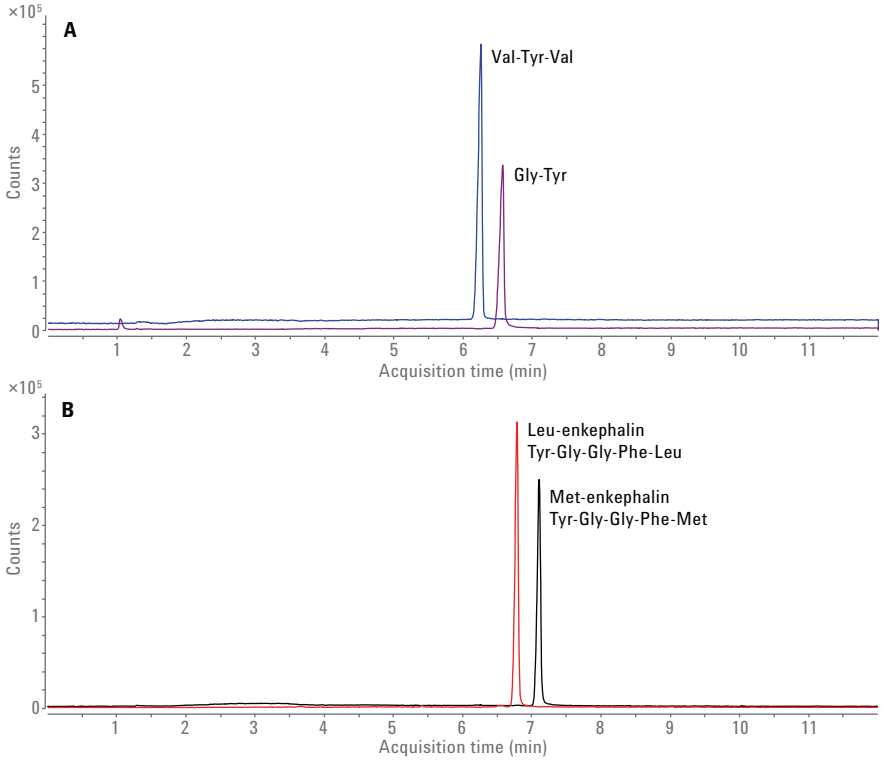

Figure 2. Separation of di- and tri-amino acids and peptides by SFC (gradient 1) and detection by SIM-MS. A) Separation of di- and tri-amino acids Gly-Tyr and Val-Tyr-Val (for abbreviations of amino acids, see Table 4). The di-amino acid Gly-Tyr elutes at 6.59 minutes and the tri-amino acid Val-Tyr-Val at 6.25 minutes with baseline separation. B) Separation of the penta-peptides Leu-enkephalin (6.78 minutes) and Met-enkephalin (7.11 minutes).

Table 2. Retention times, peak areas, and RSD values of di- and tri- amino acids and the penta-peptides Leu-and Met-enkephalin.

	Gly-Tyr		Val-Tyr-Val		Met-enkephalin		Leu-enkephalin	
	RT	Area	RT	Area	RT	Area	RT	Area
AV	6.596	1,495,481	6.254	2,338,841	7.107	892,900	6.782	1,094,260
RSD (%)	0.03	1.74	0.03	1.53	0.04	1.48	0.04	1.36

As a more complex example, the different angiotensin peptides were separated by SFC with the prior developed method. Angiotensin I is a deca-peptide that is released from the protein angiotensinogen, and has no activity itself. Further cleavage of amino acids from angiotensin I releases the most active angiotensin II and the less active angiotensin III and IV. The angiotensin peptide hormones are involved in the renin-angiotensin system, which regulates blood pressure, and is a major target for blood pressure lowering drugs. In the separation of these angiotensin peptides by SFC, angiotensin IV elutes first at 6.39 minutes followed by angiotensin III at 6.98 minutes, angiotensin II at 7.27 minutes, and finally angiotensin I at 7.41 minutes. In this separation, angiotensin II, III, and IV were clearly baseline-separated, and only angiotensin I and II showed a partial coelution due to some tailing (Figure 3). Ten replicative injections of the sample showed that the retention time and area RSDs were lower than 0.2 % and 2 %, respectively (Table 3). The separated angiotensin peptides were detected by SIM-MS with their triply- and doubly-charged ions (Table 1).

The combination of SFC with a triple quadrupole mass spectrometer, as used in this study, enables quantification of peptides, if the transitions are set up according to the target peptide. As an example, the Dynamic MRM (DMRM) and MRM method was developed for the main active angiotensin II peptide by means of Skyline software. Possible fragmentations are created by the software according to the peptide fragmentation scheme of Roepstorff and Pohlman[4] (Figure 4). A triple quadrupole acquisition method comprising these fragment masses and different collision energies for optimization was created automatically. The acquired data delivered the most intense fragments and their optimized collision energies (Figure 5A). The peptide fragmentation, all replicates of the collision energy optimization, and the automatically generated final DMRM and MRM results (Figure 5B) can be displayed at-a-glance in the Skyline software.

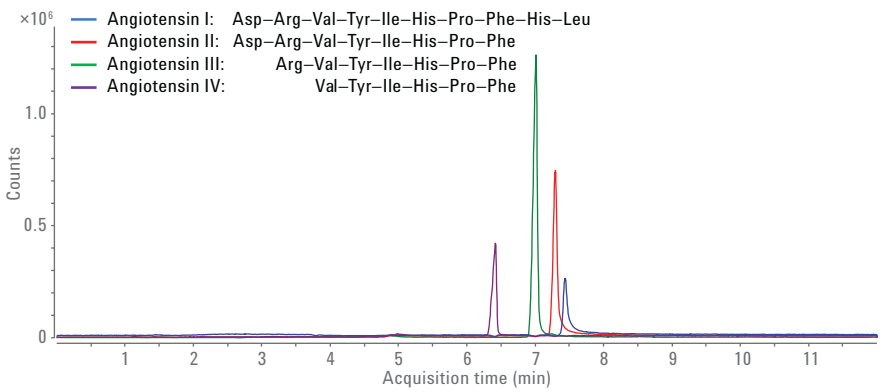

Figure 3. Separation of angiotensin I, II, III and IV by SFC (Gradient 1).

Table 3. Retention times, peak areas, and RSD values of angiotensin peptides.

	Angiotensin I		Angiotensin II		Angiotensin III		Angiotensin IV	
	RT	Area	RT	Area	RT	Area	RT	Area
av	7.415	1,544,293	7.270	3,327,340	6.988	5,476,613	6.395	1,802,408
RSD	0.15	1.91	0.16	1.83	0.19	1.43	0.19	1.10

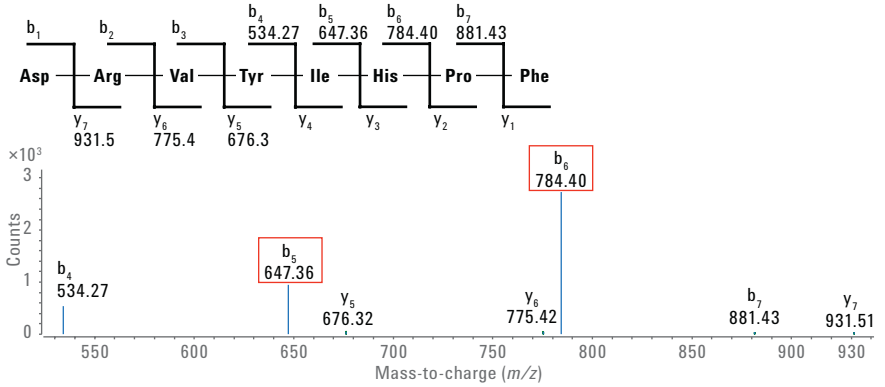

Figure 4. Fragmentation scheme of angiotensin II (MW = 1,046.2, $[M+2H]^{2+}$ = 532.7) and fragments identified from MS/MS spectra. The final DMRM and MRM method used the fragmentation 532.7 → 784.4 and 532.7 → 647.3.

Finally, as an example for a large peptide, insulin was analyzed by SFC/MS. Insulin is a peptide molecule with a molecular weight of 5,808, comprising 51 amino acids. It is built as a heterodimer of two peptide strands comprised of 21 and 30 amino acids. They are connected by two cysteine disulfide bridges (Cys—Cys). For all mammals, insulin is an important proteohormone that regulates the level of glucose in the blood. A solution of insulin in methanol/water/TFA was injected onto the 2-ethylpyridine column, and eluted as a single peak at 4.987 minutes in the middle of applied gradient 2 (Figure 6A). The extracted mass spectrum showed two charge states of $[M+4H]^{4+}$ and $[M+5H]^{5+}$ with a mass of 1,162.4 and 1,452.6, respectively (Figure 6B). After deconvolution of the charge state mass spectrum, the molecular weight of the double stranded peptide was determined to be 5,807.3 (Figure 6C).

Table 4. Proteinogenic amino acids with three-letter abbreviation and code.

Name	Abbreviation	Code
Alanine	Ala	A
Cysteine	Cys	C
Aspartic acid	Asp	D
Glutamic acid	Glu	E
Phenylalanine	Phe	F
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Lysine	Lys	K
Leucine	Leu	L
Methionine	Met	M
Asparagine	Asp	N
Proline	Pro	P
Glutamine	Glu	Q
Arginine	Arg	R
Serine	Ser	S
Threonine	Thr	T
Valine	Val	V
Tryptophan	Trp	W
Tyrosine	Tyr	Y

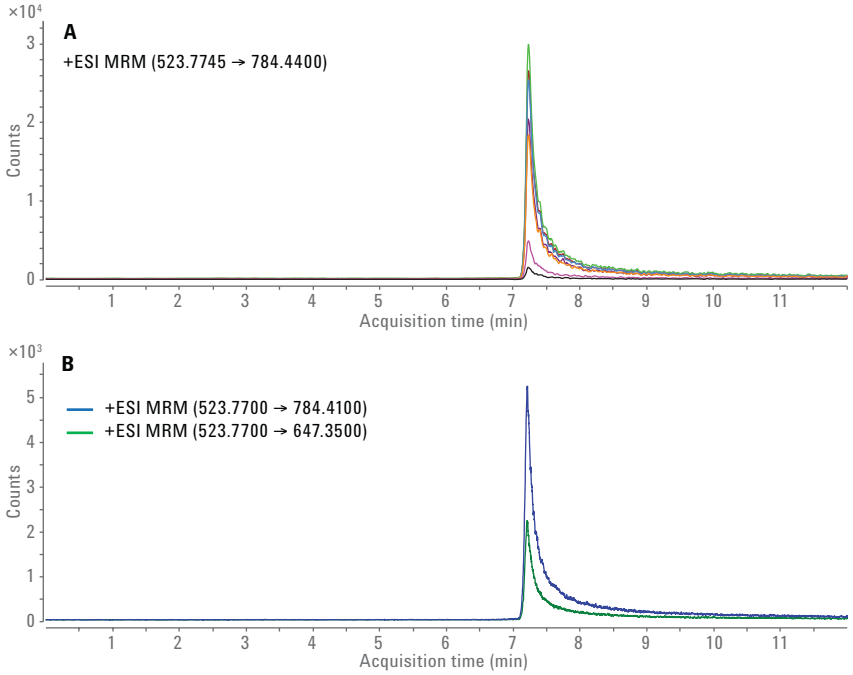

Figure 5. Collision energy optimization and final MRM measurement. A) Optimization of collision energy for the transition 523.77 → 784.44. B) Final MRM measurement with transitions 532.7 → 784.4 and 532.7 → 647.3.

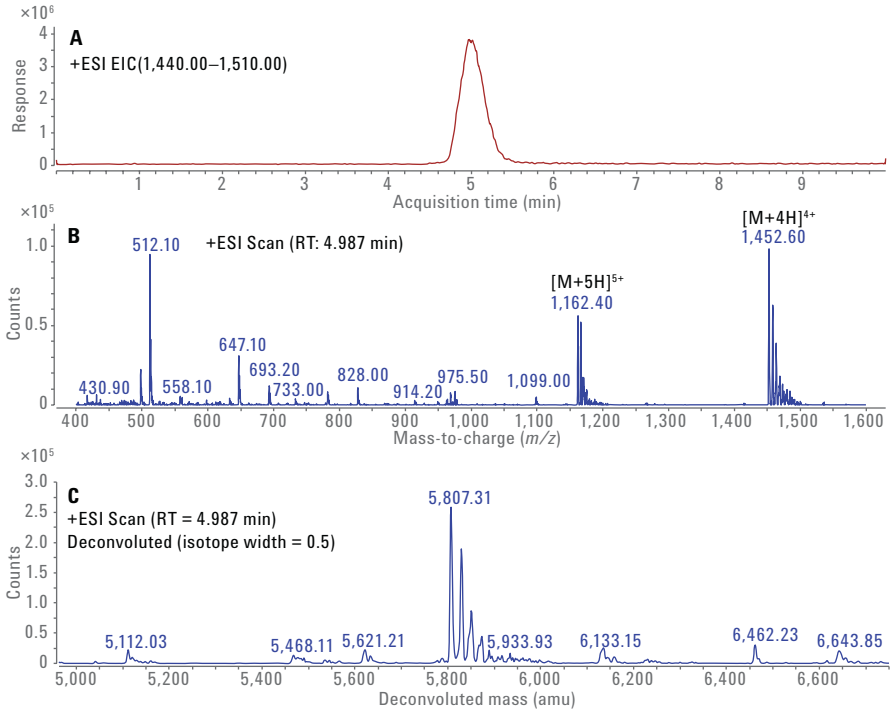

Figure 6) Analysis of insulin by SFC/MS. A) Elution of Insulin from a 2-ethylpyridine column under SFC conditions by gradient 2, extracted ion chromatogram. B) Mass spectrum of insulin at 4.987 minutes showing two charge states, $[M+4H]^{4+}$ and $[M+5H]^{5+}$. C) Deconvoluted mass spectrum of insulin, showing a molecular weight of 5,807.3.

Conclusion

This Application Note demonstrates the analysis of di- and tri-amino acids, small peptides, and large peptides by SFC/MS. The peptides are eluted in a gradient going typically up to 50 % modifier. The measured retention time RSDs are typically below 0.2 %, and the area RSDs are typically below 2 %. As an example, an MRM method was created from the octa-peptide angiotensin II with the aid of Skyline software. This demonstrates the capability of the SFC/Triple Quadrupole combination to analyze peptides. Finally, insulin, as a large peptide, was analyzed by SFC/MS and the molecular weight was determined by deconvolution of the measured charge states.

References

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