The efficient method development for the analysis of proteins, peptides, and synthetic oligonucleotides with a novel hybrid reversed phase column

Ernest J. Sobkow[1], Noriko Shoji[2], Akiko Matsui[2], Takashi Sato[2], Chie Yokoyama[2], Takatomo Takai[2], and Naohiro Kuriyama[2]

[1]YMC America, Inc., [2]YMC Co., Ltd., Japan,

Introduction

The recent development of bio-pharmaceutical industry has been remarkable and an effective analytical method with higher sensitivity, superior selectivity, and increased speed has been required in the characterization of peptides, proteins and oligonucleotides by high-performance liquid chromatography (HPLC) . The method development of reversed phase HPLC requires optimization of several conditions, such as bonded-phase, column efficiency, solvent type, pH and temperature. Especially pH and buffer type is a most important parameter to control retention of biomolecules which have multiple ionic functional groups. Furthermore, temperature often becomes a key tool to achieve better peak shapes and resolution for larger molecular-weight compounds.

Although silica based reversed phase columns have been widely used for separation of biomolecules, they have low stability under alkaline conditions and a limited usable pH range. To improve the chemical stability at an expanded pH and temperature range , we have developed a new type of organic/inorganic hybrid reversed phase column, YMC-Triart C18 and YMC-Triart C8. The novel technologies of manufacturing particles and surface modification provide outstanding chemical stability and excellent peak shape for many types of compounds under a variety of mobile phase conditions.

In this poster, we will show characteristics of this new hybrid column, and some example cases of efficient method development in separation of the biomolecules such as peptides, proteins and synthetic oligonucleotides.

Features & benefits of YMC-Triart columns

- Three core technologies for particles and surface modification

1. A multi-layered organic/inorganic hybrid particle
2. A precise granulation with microreactor technology
3. A proprietary C18/C8 bonding and a multi-stage, multi-compound end-capping

Image structure for hybrid-silica particle

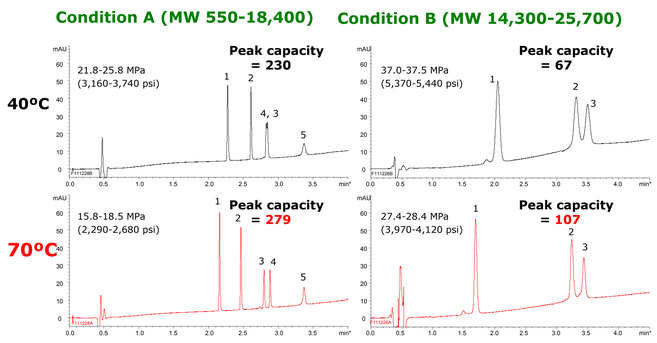
- Outstanding chemical and physical durability over a wide pH range at a high temperature
- Symmetrical peak shapes and reproducible retention for all types of compounds under a variety of mobile phase conditions
- Improved speed and resolution in UHPLC analysis on 1.9 µm columns with operating pressure up to 100 MPa (14,500 psi)
- Superior column-to-column and lot-to-lot reproducibility provided by YMC's rigorous manufacturing control system

Specification of YMC-Triart columns

Base material	Multi-layered organic/inorganic hybrid
Stationary phase	Polymerically bonded C18 group (USP L1) and C8 group (USP L7)
Particle size	1.9 µm, 3 µm, 5 µm
Pore size	120 Å
Carbon loading	C18: Approx. 20%, C8: Approx. 17%
End-capping	Yes ("multi-stage end-capping" technology)
pH range	1-12
Temperature limit (Recommendation)	70ºC for pH 1-7 50ºC for pH 7-12

Effect of increasing temperature on peak shapes and resolution in separation of peptides and proteins

Comparison of separation of peptides and proteins between at 40ºC and 70ºC

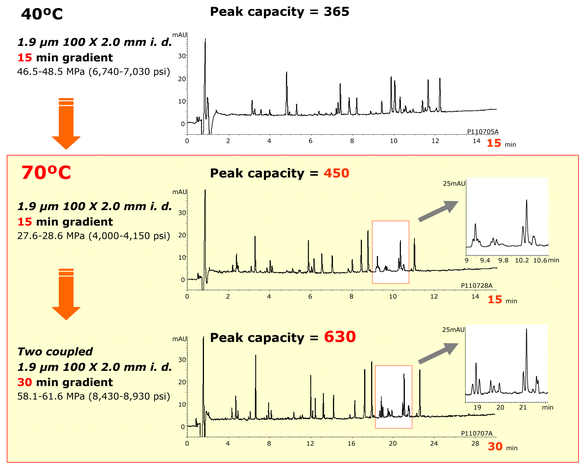


Analytes	MW	Peak width ½ (min) 40ºC	70ºC
Condition A			
1. Oxytocin	1,007	0.017	0.014
2. Leu-Enkephalin	556	0.015	0.015
3. β-Endorphin	3,465	-	0.016
4. Insulin	5,733	-	0.015
5. β-Lactoglobulin A	18,400	0.043	0.030
Condition B			
1. Lysozyme	14,300	0.069	0.044
2. α-Chymotrypsinogen	25,700	0.080	0.049
3. β-Lactoglobulin A	18,400	0.080	0.048

Column : YMC-Triart C18 (1.9 µm, 120 Å), 50 X 2.0 mm i. d.
Eluent : A) water/TFA (100/0.1)
B) acetonitrile/TFA (100/0.1) - condition A
B) acetonitrile/IPA/TFA (50/50/0.1) - condition B
Gradient : 10-80%B (0-5 min) - condition A
30-60%B (0-5 min) - condition B
Flow rate : 0.4 ml/min
Detection : UV at 220 nm
Injection : 1 µl (50 µg/ml) - condition A
1 µl (250 µg/ml) - condition B
System : Agilent 1200

- The separation of peptides and proteins with a variety of molecular weight (MW) is compared increasing column temperature from 40ºC to 70ºC, under the optimized mobile phase conditions containing 0.1% TFA.
- Although adding stronger solvent like IPA to acetonitrile of the mobile phase (condition B) is effective to reduce larger protein retention and improve peak shape, the molecules with MW >10,000 still result in peak broadening at 40ºC, as shown in the upper chromatograms.
- Increasing column temperature to 70ºC provides selectivity change, sharper peaks, and therefore, improved resolution especially for larger molecules. Generally, larger molecules diffuse very slowly compared to small molecules. An elevated temperature can improve efficiency and peak shape by lowering mobile phase viscosity and improving mass transfer, and the appropriate MW range for pore size of packing materials can be more expanded than that at a lower temperature.
- Temperature is a simple and effective tool to increase resolution in separation of proteins and peptides.

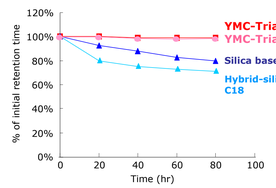
Improvement of resolution by increasing column temperature and coupling of 1.9 µm columns



- 23% more peaks can be resolved by increasing the column temperature to 70ºC in the separation of tryptic digest of Hemoglobin.
- The outstanding efficiency obtained by a coupling of two 100 mm length of Triart 1.9 µm columns reduces co-elution peaks and allows the precise separation in an analysis of complicated samples, such as peptide mapping.

Column : YMC-Triart C18 (1.9 µm, 120 Å)
Eluent : A) water/TFA (100/0.1)
B) acetonitrile/TFA (100/0.08)
5-40%B (0-15 min) for a single column
5-40%B (0-30min) for two coupled columns
Flow rate : 0.4 ml/min
Detection : UV at 220 nm
Injection : 10 µl for a single column
20 µL for two coupled columns
Sample : Tryptic digest of Bovine Hemoglobin
System : Agilent 1290

Comparison of retention stability of RP columns at pH 1 (1% TFA) and 70ºC



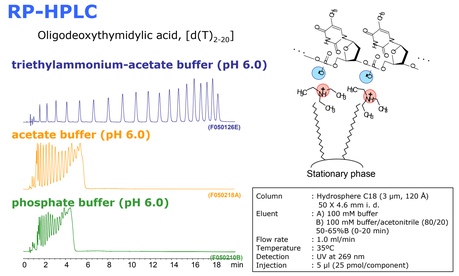
Test condition
The columns are kept in acetonitrile/water/TFA (10/90/1, pH 1) at 70ºC, and tested for performance every 20 hours under the condition of column inspection below.

[Column inspection]
Column : 5 µm, 50 X 2.0 mm i. d.
Eluent : acetonitrile/water (60/40)
Flow rate : 0.2 ml/min
Temperature : 37ºC
Sample : butyl benzoate

- Although the combination of a mobile phase containing TFA and an elevated temperature is highly effective in the analysis of peptides and proteins, the ordinary RP columns have low stability under the such accelerated condition.
- Newly developed hybrid particles and surface modification of Triart C18 and C8 provide excellent durability in the difficult conditions such as strongly acidic at elevated temperature (1% TFA, 70ºC). This advantage enables a rapid and efficient method development of a complex mixture of peptides and proteins.

Effective separation of oligonucleotides by using an ion-pairing mobile phase and optimizing a temperature

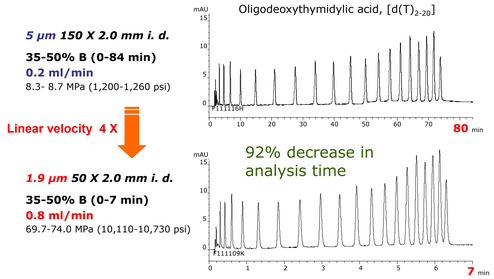
Mobile phase composition and retention mechanism of oligonucleotides in RP-HPLC



Column : Hydrosphere C18 (3 µm, 120 A) 50 X 4.6 mm i. d.
Eluent : A) 100 mM buffer
B) 100 mM buffer/acetonitrile (80/20)
50-65%B (0-20 min)
Flow rate : 1.0 ml/min
Temperature : 35ºC
Detection : UV at 269 nm
Injection : 5 µl (25 pmol/component)

- The separation of oligodeoxythymidylic acids, $d(T)_{2\text{-}20}$ is compared among the triethylammonium-acetate (TEAA), ammonium acetate and potassium phosphate buffers under the same gradient condition. The retention achieved with the TEAA buffer was stronger than that achieved with the acetate or phosphate buffer.
- The ion-pairing reagent containing both a positively charged group and a hydrophobic functional group, such as triethylamine (TEA) and dibutylamine (DBA) can interact with the negatively charged phosphodiester groups of oligonucleotides and also the hydrophobic stationary phase. It results in stronger retention and superior resolution of oligonucleotides.

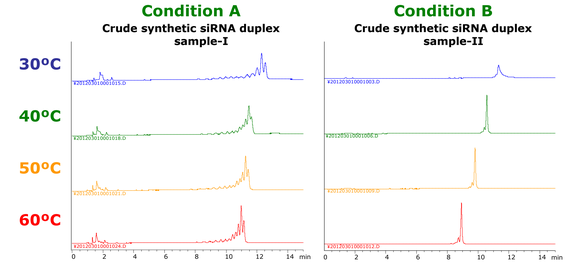
Increase throughput with 1.9 µm column and higher flow rate



Column : YMC-Triart C18
Eluent : A) 10 mM di-n-butylamine-acetic acid (pH 6.0)
B) methanol
Detection : UV at 269 nm
Temperature : 35ºC
Injection : 1µl (5 nmol/ml)
System : Agilent 1290

- The conventional HPLC method is transferred from 5 µm 150 X 2.0 mm i. d. to 1.9 µm 50 X 2.0 mm i. d. in the separation of oligodeoxythymidylic acids, $d(T)_{2\text{-}20}$. The identical selectivity of Triart columns across different particle sizes provides the predictable method transfer without any change of elution profile.
- 19 peaks are completely resolved within 7 minutes using 1.9 µm UHPLC column at four times faster linear velocity.

Effect of column temperature on separation of oligonucleotides

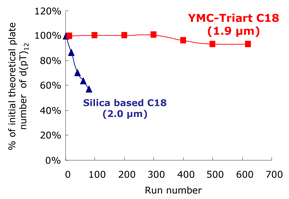


Sample-I: synthetic siRNA duplex (19 bp)
5'-CUG GAC UUC CAG AAG AAC AdTdT-3'
3'-dTdTGAC CUG AAG GUC UUC UUG U-5'

Sample-II: synthetic siRNA duplex (19 bp)
5'-CGU ACG CGG AAU ACU UCG AdTdT-3'
3'-dTdTGCA UGC GCC UUA UGA AGC U-5'

Column : YMC-Triart C18 (1.9 µm, 120 Å), 100 X 2.0 mm i. d.
Eluent : A) 10 mM di-n-butylamine-acetic acid (pH 6.0)
B) acetonitrile – Condition A
B) methanol – Condition B
Gradient : 20-45%B (0-15 min) – Condition A
35-60%B (0-15 min) – Condition B
Flow rate : 0.2 ml/min
Detection : UV at 269 nm
Injection : 1 µl (5 nmol/ml)
System : Agilent 1290

Durability at pH 6.0 (DBAA buffer) and 65ºC



Test condition
Column : 1.9 µm or 2.0 µm, 120 Å, 50 X 2.0 mm i. d.
Eluent : A) 10 mM di-n-butylamine-acetic acid (pH 6.0)
B) methanol
30-50%B (0-20 min)
Flow rate : 0.4 ml/min
Detection : UV at 269 nm
Temperature : 65ºC
Sample : Oligodeoxythymidylic acid, $[d(T)_{2\text{-}20}]$
Injection : 1 µl (5 nmol/ml)
System : Agilent 1290

- In non-denaturing HPLC analysis of crude synthetic siRNA duplex, increasing temperature improves the peak shape and the resolution between the target compound and the impurities as shown in above chromatograms.
- The ordinary silica based RP columns have lower stability under the suitable conditions for oligonucleotides analysis, such as at a neutral pH and a higher temperature. Triart C18 demonstrates outstanding stability over repeated injections at 65ºC.

Conclusions

- Highly sensitive, selective and reproducible HPLC methods can be developed with a novel reversed phase hybrid column using pH and temperature as key tools for optimization, in the analyses of peptides, proteins, and oligonucleotides.
- YMC-Triart columns offer significant advantages for simple and rapid method development of a variety of biopharmaceutical compounds.