Technical Notebook

Amino Acids, Peptides, Proteins

Amino acid (1)

Amino acid (2)

N-terminus  C-terminus

Number 3
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Introduction

The analysis of Amino Acids, Peptides, and Proteins are essential to a multitude of fields: food science, biomolecular studies, and pharmaceutical development to a highlight few. High Performance Liquid Chromatography (HPLC) can play a vital analytical role across all these fields due to the diversity of separation techniques available to chromatographers.

Chromatographic Conditions and Column Selection

This technical notebook covers some of the most chemically and physically diverse compounds in the new technical notebook series. For example, some peptide separations may involve numerous compounds that are within tens of Daltons in weight difference. It is important to review the differences in polarity and molecular weight as well as solubility when choosing the best column for the desired separation. There is no one “exact” column that can be used. However, a few general principles can help avoid time-consuming method development or altogether failure of the separations. Please refer to Technical Notebook 1 for an extensive discussion on separation modes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>Mode(s)</th>
<th>Examples</th>
<th>Notes</th>
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<tbody>
<tr>
<td>A</td>
<td>High MW, Low polarity</td>
<td>SEC (organic GPC)</td>
<td>Plastics, cellulose, some proteins</td>
<td>Olefins, CMC</td>
</tr>
<tr>
<td>B</td>
<td>High MW, High polarity</td>
<td>SEC (aqueous GFC), ion exchange, multimode</td>
<td>Proteins, some PEG-PEO, some polysaccharides</td>
<td>Polysaccharides depend on linkages and sugar types</td>
</tr>
<tr>
<td>C</td>
<td>Low MW, Low polarity</td>
<td>Reversed phase (moderate polarity), Normal phase (NP)</td>
<td>Small organic compounds (alkane derived)</td>
<td>Hydrocarbons, NP: special fatty acids and fat-soluble vitamins</td>
</tr>
<tr>
<td>D</td>
<td>Low MW, High polarity</td>
<td>Normal phase, ion exchange, HILIC, IC, IEC, SEC, RP, multimode</td>
<td>Metal ions, small polar sugars, oligosaccharides</td>
<td>HILIC (Hydrophilic Interaction Liquid Chromatography) is a special form of NP</td>
</tr>
</tbody>
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*Figure 1: Figurative graph of separation mode by molecular weight vs solvent polarity*

*Figure 2: Diagram showing the separation modes for different molecular weights and polarities.*
Proteins and peptides are commonly studied in biotechnology, pharmaceuticals and food science. HPLC is one of the most common analytical methods for proteins or amino acids. The main separation modes used for protein analysis are gel filtration chromatography (size exclusion chromatography), reverse phase chromatography, ion exchange chromatography, hydrophobic interaction chromatography, affinity chromatography, etc. For analysis of peptides and amino acids, a combination of HILIC mode and LC/MS can also be used. Figure 4 depicts a options of different analysis techniques.
Figure 4
Amino Acids
The key functional groups composing an amino acid are an amine group, a carboxyl group, and side chain unique to each acid. The “charged groups” and polarity of the side chain allows for Amino Acids to separated using a variety separation techniques detailed below.

HILIC

![Simultaneous HILIC – MS Analysis of Amino Acids (VG-50 v VC-50)](image)

**Figure 5: Amino Acids via HILIC**

HILIC columns can retain polar compounds, with this ability, they can separate amino acids based on their relative polarity. Amino acids are amphoteric, allowing control of elution by modifying the pH as seen in Figure 5. While the HILICpak VG-50 stationary phase is slightly more polar than HILICpak VC-50, the change in elution order is due to the pH of the eluent. The VG-50 separation used a basic eluent, leading to increased retention of the aspartic acid and glutamic acid. This is reversed with acidic eluent in the VC-50 separation as the basic amino acids, Histidine and Arginine, are retained longer. All columns in the HILICpak series are optimized for highly sensitive detectors including MS.

Reversed Phase
Relative polarity of the eluent, as with HILIC, drives reverse phase separations of amino acids but ionic interactions also play a large role.
As RSpak NN-814 is packed with polyhydroxymethacrylate functionalized with sulfo groups, the main separation mode is the reversed phase mode. However, due to the sulfo groups, ion interaction also works. Acidic amino acids, such as aspartic acid, are separated by ion exclusion mode and elute faster. Neutral substances are separated by reversed phase and alkaline amino acids are separated by a mixture of reversed phase (in case of hydrophobic structure) and ion exchange mode. Therefore, amino acids basically elute in the following order: first the acidic amino acids, second the neutral amino acids and finally the alkaline amino acids.
YS-50 separates amino acids using a combination of reversed phase and ion exchange as depicted above.

**Peptides**

**Multimode**

The Shodex Asahipak GS-HQ series mainly relies on size exclusion chromatography but also works in multimode with the combination of reversed phase and ion exchange modes. Different separation patterns from standard size exclusion chromatography should be expected. The GS-HQ series is particularly suited for the separation of hydrophilic peptides, especially acidic peptides and basic peptides. The base material is a polymer, resulting in the following advantages; usable at a wide pH range, washable with alkaline solutions, column durability.

As seen above, this peptide separation a variety of separation modes are required as SEC, RP, or IEX cannot achieve the separation alone. The GS series offers chromatographers a specialty tool for difficult separations.
Reversed Phase
A sample containing various peptides was separated using an eluent where the pH values changed. Under acidic conditions (pH2.3, pH3.0) Leu-Enkephalin and Leu-Trp-Met were strongly retained and the elution times are quite similar; resulting in an unsatisfactory separation. Under alkaline condition (pH10) a better separation with sharper peaks of the substances has been obtained in a shorter time. In addition, ODP columns have higher theoretical plate numbers under alkaline conditions.

Effect of pH on Peptide Analysis (OPD Series)

SEC
Various peptides with MW ranged from 269 to 1734 was analyzed using aqueous solvent SEC (GFC) high performance semi-micro column, KW402.5-4F. Since each peptide may contain different amino-functional groups, the separation mode occur may not be as a “pure” size
exclusion mode like for the analysis of proteins. This is more significant when peptides contain more hydrophobic-amino acids and/or basic-amino acids.

HILIC
The imidazole dipeptides, anserine (dipeptide of histidine and β-alanine) and carnosine (dipeptide of 1-methylhistidine and β-alanine), are found in large concentrations in the meat of bonito, tuna, whale, and the breast meat of birds. The imidazole dipeptides show a strong antioxidant effect which is effective for reducing fatigue and anti-aging. In this application, carnosine, anserine, and their constituent amino acids were analyzed simultaneously using polymer-based amino column, Asahipak NH2P-50 4E. These five compounds can be separated completely within 20 minutes.
Mixed Samples – Peptides and Proteins

The ODP series features a pore size of 250 Å and can be used for both proteins and peptides. For larger proteins, the RSpak RP18-415 is recommended with a pore size of 450 Å.
The ODP series are packed with a polyvinyl alcohol polymer base offering excellent durability. The series further excels for proteins and peptide separations by maintaining consistent retention times and recovery throughout the columns lifetime. The polymer base avoids unwanted protein and packing material interactions.

Proteins
Size Exclusion
The packing material of the Shodex PROTEIN KW-800 series is composed of silica particles whose surface is coated with a hydrophilic polymer. This column is for aqueous SEC, and indexed in the Shodex catalogue under gel filtration chromatography (GFC). The PROTEIN KW series is offered in three column diameters, differing in pore and particle size.
The Shodex KW400-4F series is the high-performance semi-micro column version of the KW-800 series. The theoretical plate number and the sensitivity are improved by reducing the diameter of the packed particle to 3 micrometers. KW405-4F, the newest addition to the KW400 line-up, has a larger pore size than any columns in the existing KW-800 series, and can analyze proteins whose molecular weight is larger than 1,000,000 Da.

Modified Proteins
PEGylation of drugs and proteins have become an important method to improve several pharmacological parameters. Increased MW, solubility, and stability all come with challenges for HPLC separations and quality monitoring. Above, PEG modified chymotrypsin is analyzed using PROTEIN KW-802.5.

\[ \alpha, \text{ Acid Glycoprotein – Orosomucoid (KW-804)} \]
Glycoproteins, including antibodies, have also become major areas of interest for pharmaceutical research. Bonding to the packing material can cause problems for other GFC columns but the high quality and consistent polymer coating of the KW series allows for repeatable separations and recoveries.

Antibodies

Antibody drug conjugates are an important class of highly potent biopharmaceutical drugs designed for targeted therapy. Antibody drugs work with the immune system, limiting side effects relative to conventional drugs. Quality control, specifically aggregate and fragment concentration, are important and have potential impact on pharmacological activity and safety. The LW series have been specifically optimized for antibodies including IgG. The LW series also has increased theoretical plates, and ultra-low bleed for clean light scattering baselines.

Ion exchange chromatography

Ion exchange chromatography is an analytical method that relies on the interaction of charged ions on the surface of proteins with the surface of the column packing material. Packing materials with a positive surface charge are called “anion exchangers” and those with a negative surface charge are called “cation exchangers”. Obtaining some information about the isoelectric point (pI) of proteins is useful for selecting suitable analytical conditions. That pH is called isoelectric point (pI). Around the pI, the electrical charge on the surface of proteins is neutralized and such proteins cannot easily adsorb on the surface of the packing material. In some cases, proteins precipitate around the pI, a phenomenon known as “isoelectric precipitation”. In order to adsorb the target protein, the pH of the eluent must be adjusted at more than 1.0 pH unit from pI. When the pH is
more acidic than the pI, a cation exchanger should be used, and when the pH is more alkaline than the pI, an anion exchanger should be selected.

In the case of the separation of a protein whose pI is 7, the pH of the eluent should be adjusted to 8, to make the protein anionic. The DEAE ion exchanger should be equilibrated with a buffer solution with chloride (Cl-) as the counter ion. When the protein, which has negative charge as an anion, is introduced into the column, it binds to the DEAE ion exchanger while removing Cl-. If a protein with a pI of 9 is mixed in the sample, that protein exists as cation and goes through the DEAE ion exchanger without adsorption. In order to elute the adsorbed proteins, there are two methods. One elution method is the gradual concentration increase of salts such as sodium chloride (NaCl) in the eluent, and another method is the gradual change of pH in the eluent. The first method is used more prevalently.

When the concentration of NaCl increases in the eluent, the concentration of Cl- increases and competes for adsorption against previously adsorbed negatively charged proteins, which can remove proteins from the anion exchanger. The situation of the separation also depends on the type of ion exchanger. An ion exchanger is classified not only by anion or cation exchanger type, but also by the strength of the exchanger resulting from differences in functional groups. The difference between a strong and a weak exchanger is not a difference in the binding strength for proteins but the difference in the effect of pH change in the eluent, i.e. the difference in buffering capacity. Strong ion exchangers can keep the same electrical charge and adsorb proteins in a wider pH range, while week ion exchangers are affected by pH changes in the eluent, readily changing the ion exchange capacity.

A weak anion exchange resin with a DEAE functional group is packed into IEC DEAE-825 and Asahipak ES-502N columns. These columns are suitable for the separation of acidic proteins because the pKa of the ion exchanger is about 7.8.
For fast analysis, columns packed with a non-porous gel are effective. A non-porous gel is made of packing material that does not have pores. The particle diameter of a non-porous gel can be shortened and the compressive strength of the gel is high, so it can be used for fast analysis with increased eluent flow rates. The analytical time with DEAE3N-4T is shorter than the time with IEC DEAE-825, which is packed with a porous weak anion exchange resin.

**Maximum flow rate**
- DEAE-825, SP-825 (porous resin): 1.5ml/min
- DEAE3N-4T, SP-420N (non-porous resin): 2.0ml/min

**Maximum protein loading volume**
- DEAE-825, SP-825 (porous resin): 1,000ug/column
- DEAE3N-4T, SP-420N (non-porous resin): 20ug/column

The figure above shows the effect of pH on the separation of proteins on weak anion exchange chromatography column IEC DEAE-825. Under acidic eluents, the four kinds of protein can be separated well but under alkaline eluents, the separation worsens with increasing pH. Weak anion exchangers (DEAE) are preferably used for protein analysis, even though the pH range for protein retention is narrower than the range for a strong anion exchanger, because milder analytical conditions can be used.
Reversed Phase

The separation mode of reversed phase chromatography is based on the hydrophobic interactions governing the distribution/adsorption equilibrium between nonpolar amino acids in proteins and the surface of the packing material. The surface of the packing material's gel is bound with a hydrophobic functional group such as octadecyl (C18). Some polymer gels can be used for reversed phase chromatography without the need for a functional group because the base material in itself is hydrophobic. Proteins with a higher hydrophobicity are retained strongly, so hydrophilic proteins are eluted initially.

The chromatogram and recovery rate of proteins with the polymer-based reversed phase chromatography column RSpak RP18-415 are shown above. The figure shows that the proteins and peptides are analyzed in 25 minutes with a high recovery rate. The maximum durable pressure is 22 MPa, so the flow rate can be increased for faster analysis if necessary.
HIC
The mechanism of hydrophobic interaction chromatography (HIC) is based on the hydrophobic interaction between proteins and the surface of the packing material. It is similar to reversed phase chromatography with different eluent conditions. As the salt concentration increases, the hydrophobicity of the protein surface also increases and the protein adsorbs to the packing material. This effect is similar to a “salting out” method, a procedure usually carried out with ammonium sulfate. After adsorption, the adsorbed protein is eluted by decreasing the salt concentration thus weakening the hydrophobic interaction with the packing material. The protein is adsorbed under high salt concentration, so this separation mode is usable after ammonium sulfate precipitation or ion exchange chromatography. A less hydrophobic base is used as the functional group, hence the analytical conditions for proteins are milder than the conditions of reversed phase chromatography, preserving the bio-activity of protein. Shodex HIC PH-814 is a column for hydrophobic interaction chromatography, with a phenyl group bound to the packing material as a hydrophobic ligand.
Nucleosides and nucleotides

For the chromatography challenges that nucleosides and nucleotides present, the multimode GS series allows the user to combine size exclusion with reverse phase and ion exclusion chromatography to tackle these separations.