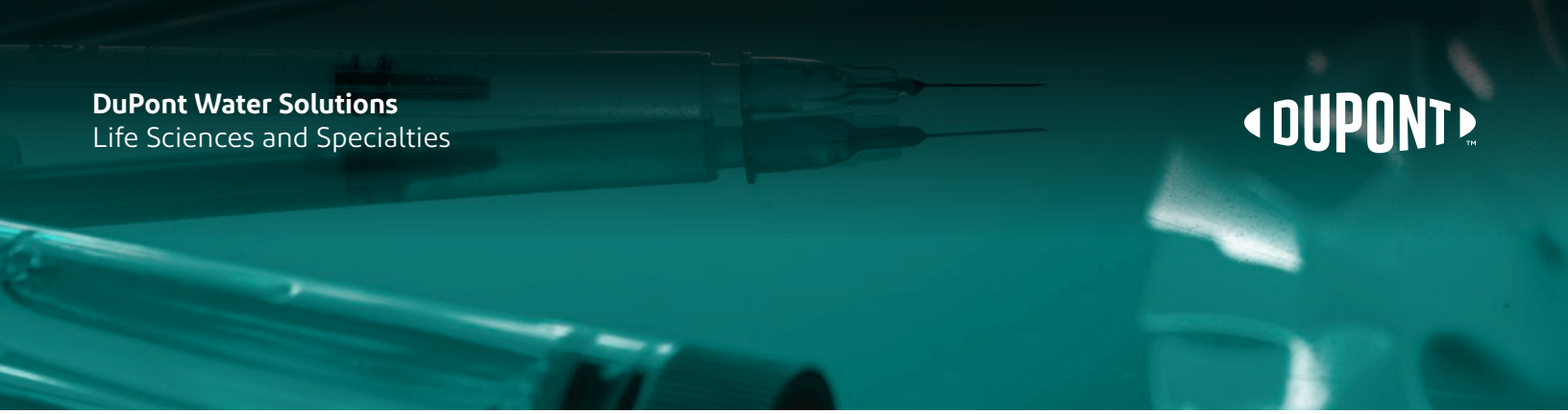

DuPont Water Solutions
Life Sciences and Specialties

Peptide Separation with DuPont™ AmberChrom™ Chromatography Resins

Introduction

The field of peptide therapeutics has grown significantly since the discovery of insulin around a hundred years ago. Modern peptides are produced through various routes – synthetic, recombinant, or enzymatic. Synthetic routes allow more modifications as well as the use of non-natural amino acids, but they have limitations since inefficient chemical coupling can decrease the overall yield as the number of subunits increase. Recombinant routes can be used in more challenging peptides, especially when post-translational modifications are required, while enzymatic routes are favored in the production of short peptides.[1] Regardless of the production route, the crude product must be purified to obtain the high quality demanded for drug substances.

Peptide purification relies on resin interactions with peptide amino acid side chains and charges. The dual nature of the amino acids gives peptide manufactures two chromatography routes for peptide purification: ion exchange chromatography and reverse phase chromatography (RP-HPLC). The former relies on ionic interactions and the latter relies on hydrophobic interactions. Both approaches are commonly used for peptide purification, often in the same purification train as complementary techniques. Within RP-HPLC, downstream purification engineers have the choice of two classes of chromatography resins. Silica resins and polymeric resins both have long-standing history as RP-HPLC resin media for peptide purification. This paper compares polymer resins and silica resins for peptide reverse phase chromatography using a model peptide mixture to show the ability of each system to separate peptides with slightly different chemical structures and to illustrate the role of surface area in peptide separations.

Peptide Purification by Reverse Phase Chromatography

Principles of Separation

RP-HPLC uses a nonpolar resin with a polar mobile phase. The peptides adsorb to the stationary resin surface through hydrophobic forces and elute from the resin in order of hydrophobicity by passing a mobile phase with increasing concentrations of organic solvent [2] through the column. With this scheme, more hydrophobic peptides elute later in the gradient, as they have stronger interactions with the hydrophobic resin phase.

Most peptide purifications use a combination of water and a solvent, such as acetonitrile, as the mobile phase, along with an acid modifier such as trifluoroacetic acid (TFA) or formic acid. These acid modifiers decrease the peptide charge density and increase the hydrophobic interactions with the resin by protonating the acidic functional groups, which can interfere with the adsorption on the resin. Formic acid, however, has advantages in that it is not as harsh as TFA, the chemical structure does not contain fluorine, and it is more compatible with analytical detection methods.

In this study, the peptide separation performance of polymeric resins from the DuPont™ AmberChrom™ chromatography resin family (Table 1) is compared to commercial C18 silica using formic acid as the acid modifier. A commercially-available peptide test mixture was obtained from Sigma Aldrich (Table 2). The mixture contains peptides ranging from 2 to 8 amino acid subunits and is used to demonstrate the ability to isolate peptides with different amino acid structures. The peptides are expected to elute in order of amino acid hydrophobicity, which will increase with the hydrophobicity of the side chains and their number. The first two peptides are expected to elute early as they contain only a couple of hydrophobic side chains. The two Enkephalin peptides should elute later as they contain more hydrophobic amino acids; the leucine-modified sequence is expected to be slightly more hydrophobic and elute as the fourth peak.[2] Finally, the Angiotension peptide is expected to elute last as it contains the hydrophobic amino acids valine, tyrosine, isoleucine, and phenylalanine. The last three molecules could exhibit co-elution if the mobile phase conditions drive similar hydrophobic interactions with the resin.

Table 1. DuPont™ AmberChrom™ XT chromatography resins and DuPont™ AmberChrom™ CG chromatography resins used in this study. All are based on crosslinked divinyl benzene.

Grade	Particle size, mean	Pore size, average (Å)	Total Surface Area (m^2/g)
XT20	20 µm	300	560 - 580
CG161S	~35 µm	150	900
CG300S	~35 µm	300	700

Table 2. Five-peptide mixture used in this study.

Peptide	MW (Da)	Elution Order
Gly-Tyr	238.2	Peak 1
Val-Tyr-Val	379.5	Peak 2
Methionine Enkephalin acetate Tyr-Gly-Gly-Phe-Met	573.7	Peak 3
Leucine Enkephalin Tyr-Gly-Gly-Phe-Leu	555.6	Peak 4
Angiotension II acetate Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	1046.2	Peak 5

Gly = glycine; Tyr = tyrosine; Val = valine; Phe = phenylalanine; Met = methionine; Leu = leucine; Arg = arginine; Ile = isoleucine; His = histidine; Pro = proline

Materials and methods

The HPLC peptide standard mixture was from Supelco™ and purchased from MilliporeSigma™ (H2016). It was provided in a dried film with 0.5 mg of each component. Peptide samples were prepared at 2 mg/mL by dissolution in ultrapure water. Acetonitrile (Fisher Scientific™) and formic acid (Thermo Scientific™) were used as purchased to prepare the mobile phases. Mobile phase A consisted of 0.1% formic acid in 5:95 acetonitrile:water and mobile phase B consisted of 0.1% formic acid in 75:25 acetonitrile:water. Samples were held in 2 mL vials with low-volume inserts at 5°C before runs which were performed on an Agilent™ 1260 Infinity II HPLC system equipped with UV detection. Detection was done at 214 nm.

The gradient was usually kept the same (5 – 30%) while changing the number of column volumes (CVs), i.e. changing the slope of the same gradient as the run length changes, with CV being 4.15 mL for all the columns used here (column dimensions: 4.6 x 250 mm).

Resins used: DuPont™ AmberChrom™ XT20 chromatography resin, DuPont™ AmberChrom™ CG161S and CG300S chromatography resins (see Table 1 for resin properties), and a commercially available C18 silica resin with an average particle size of 16 µm and a specified pore size of 300 Å.

Results

DuPont™ AmberChrom™ XT20 chromatography resin was compared to a silica resin with a 16 µm particle size at the same 300 Å pore size using formic acid as the acid modifier in gradient elution over 6 column volumes (CV). As expected, the silica resin, with its smaller diameter, gave sharper peaks but the 20 µm AmberChrom™ XT20 chromatography resin separated even the highly similar peptides that differ in a single amino acid (peaks 3 and 4, Figure 1a-b), despite its larger particle size. There was a slight shift to longer elution times of peaks 2 – 5 on C18 silica when compared to the polymeric resin, which is attributed to increased interactions between the peptide and the silica resin. In a scaled-up manufacturing operation, it is desirable to have earlier elution with fewer column volumes because it requires less solvent usage.

The impact of pore size on the separation of these peptides was evaluated on DuPont™ AmberChrom™ CG161S and CG300S chromatography resins. Figure 2 shows that AmberChrom™ CG161S resin gave higher resolution than AmberChrom™ CG300S resin. These resins have the same mean particle size of 35 µm but the total surface area, as measured by N_2 BET, is higher in AmberChrom™ CG161S due to the smaller pore size (Table 1). The peptide-resin interaction time increases with surface area, so AmberChrom™ CG161S exhibited improved hydrophobic-driven separation.[3] DuPont™ AmberChrom™ CG300S was not able to fully resolve peptides 3 and 4, but DuPont™ AmberChrom™ CG161S resin was able to resolve these closely-related peptides, despite the relatively large 35 µm particle size, because of the large surface area available in this polymeric resin.

Conclusions

RP-HPLC is widely used in this field due to its effectiveness in separating peptides with similar chemical identities. Polymeric resins, which have inherently better pH stability and less complicated surface chemistries, are capable of separating peptides with subtle differences in structures. In this paper, the polymeric resins were able to resolve peptides that differed in a single amino acid. There are many resin design elements that can impact purification performance. Please contact DuPont TS&D for help in selecting a resin for your system.

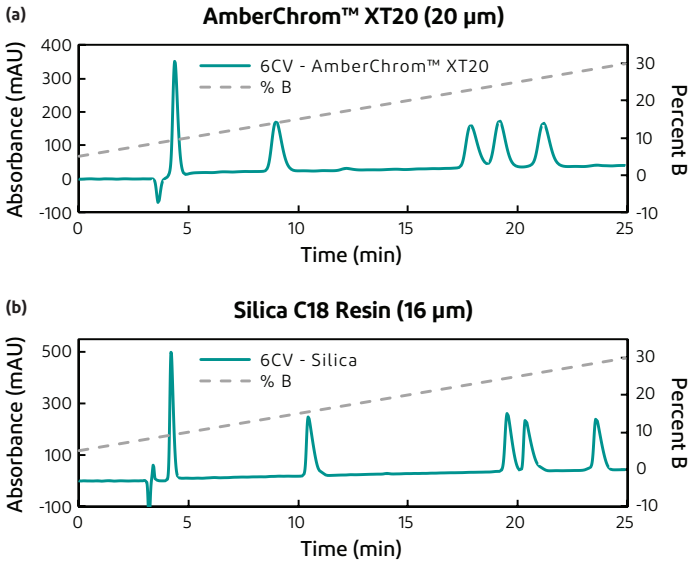

Figure 1. Elution of the 5-peptide mixture in 5 – 30% buffer B with formic acid on **(a)** AmberChrom™ XT20 chromatography resin and **(b)** 16 µm C18 silica resin.

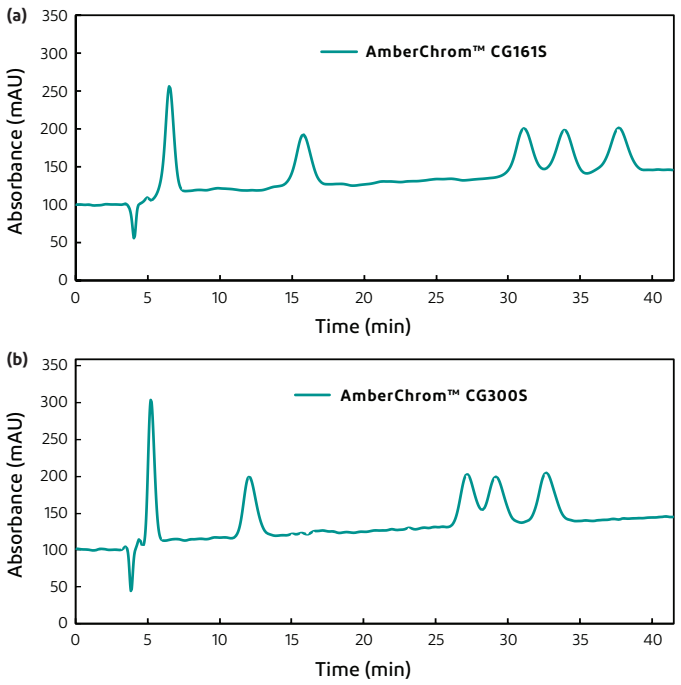

Figure 2. Elution of the 5-peptide mixture in 5 – 30% B using the same gradient over 10 CVs on **(a)** DuPont™ AmberChrom™ CG161S chromatography resin and **(b)** DuPont™ AmberChrom™ CG300S chromatography resin.

References

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