

Separate and Purify Pharmaceuticals and Small Biomolecules, Using Polymeric RPLC Resins

Amberchrom resins bring unique advantages to small molecule separation and purification procedures. They frequently are the only materials that can remove specific compounds from the eluant. Excellent physical, chemical, and thermal stability makes these materials highly versatile.

Key Words:

- antibiotics purification • pharmaceuticals purification
- peptide purification • small protein purification

High performance reversed phase liquid chromatography (RPLC) is the technique of choice for analyses of small molecular weight compounds (<20,000 Daltons) in the pharmaceutical and chemical industries, and biochemical research. Traditionally, silica-based packings have been the most commonly used sorbents in RPLC. For proteins and peptides, however, the unique properties of resin-based packings (e.g., physical and chemical stability) have been used to advantage in many applications (Table 1).

Table 1. Small Biomolecules Separated/Purified Using Amberchrom Resins

| | |
|-----------------------|--------------------|
| Antibiotics | Synthetic Organics |
| Oligonucleotides | Vaccines |
| Peptides & Proteins | Vitamins |
| Phytopharmacologicals | |

The Amberchrom® line of styrenic (Amberchrom CG-161, CG-300, CG-1000) and methacrylic ester-based (Amberchrom CG-71) polymeric resins are well suited for process scale RPLC. These resins are chemically, mechanically, and physically stable. They are resistant to microbial attack, dimensionally stable to changes in pH and ionic strength, and compatible with a variety of organic solvents. They can be sterilized by autoclaving (although this might reduce protein uptake) and cleaned by treatment with 0.5N sodium hydroxide. We offer Amberchrom resins in three particle size ranges (20-50µm, 50-100µm, and 80-160µm) for optimization of efficiency.

Physical properties of the Amberchrom resins are shown in Table 2. Polymeric resins have consistent properties from the surface of the bead to the center. Since they are stable to strong acid or base, retention behavior is unchanged after many clean-in-place operations (Figure A). Amberchrom resins exhibit relatively small changes in bed volume with a broad range of solvents (Table 3).

Advantages of Using Amberchrom Resins

Amberchrom resins are smaller particle versions of Amberlite® XAD® adsorbent resins, designed for higher performance purifications that are not possible with the larger XAD resins. For example, Amberchrom CG-161 provides greater resolution of a dye mixture than Amberlite XAD-16 (Figure B).

Table 2. Physical Properties of Amberchrom Resins

| Resin | Surface Area (m ² /g) | Porosity (Vol. %) | Mean Pore Size (Å) | Grams Dry Polymer/Wet mL |
|---------|----------------------------------|-------------------|--------------------|--------------------------|
| CG-71 | 500 | 58-63 | 250 | 0.23 |
| CG-161 | 900 | 65-69 | 150 | 0.25 |
| CG-300 | 700 | 55-75 | 300 | 0.21 |
| CG-1000 | 250 | 65 | 1000 | 0.23 |

Figure A. Amberchrom Resins Cleaned with NaOH

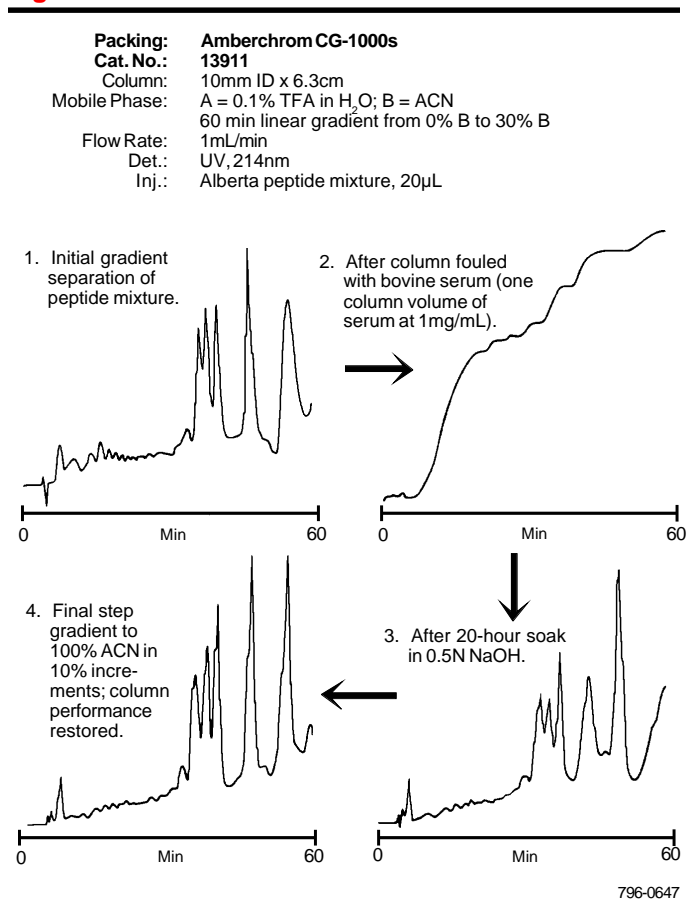


Table 3. Percent Swelling of Amberchrom Resins in Solvents

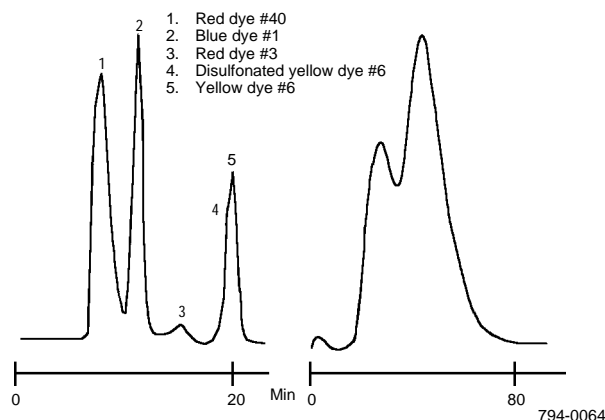
| Resin | Acetone | Isopropanol | Methanol | Toluene | Water |
|--------|---------|-------------|----------|---------|-------|
| CG-300 | 4 | 4 | 3.5 | 5 | 0 |
| CG-161 | 14 | 12 | 8 | 4 | 0 |
| CG-71 | 6 | 7 | 2 | 5 | 0 |

Figure B. Amberchrom Resins Provide Higher Resolution than Traditional, Larger Particles

Columns: 10mm ID x 25cm
 Mobile Phase: water/0.1% TFA to 100% acetone in
 20 min (A) or 80 min (B)
 Flow Rate: 0.5mL/min loading, 5.0mL/min gradient
 Det.: VIS
 Inj.: alura red

Amberchrom CG-161m (50-100µm particles)

Amberlite XAD-16 (297-840µm particles)



The unique surface chemistries and physical properties of Amberchrom resins ensure selectivity and large capacities for separating and purifying biomolecules and pharmaceuticals (Table 4). As a result, these resins:

- allow use of aggressive mobile phases (pH range 1-14) for selectivity optimization
- allow gradient elution or cleaning with a broad range of solvents, with minimal change in bed volume
- allow use of high flow rates, for high throughput with moderate backpressure
- can be cleaned in place easily with strong acid or base, which allows endotoxin removal
- can be thermally or chemically sanitized (temperatures above 50°C might reduce protein capacity)
- enable repeated cycles with assured column stability and reproducible performance

Table 4. Saturation Capacity (mg/mL) of Amberchrom Resins

| Resin | Ceph C (490 Da) | Vancomycin (1485 Da) | Insulin (5733 Da) | BSA (67,500 Da) |
|-----------------|--|-------------------------|----------------------|--------------------|
| Styrenic | | | | |
| CG-161 | 107 | 87 | 91 | 43 |
| CG-300 | 72 | 50 | 97 | 74 |
| CG-1000 | 27 | 25 | 38 | 36 |
| Acrylic | | | | |
| CG-71 | 27 | 35 | 98 | 36 |
| Column: | 10mm ID x 6.3cm | | | |
| Elution: | cephalosporin C — 10% IPA / 90% water vancomycin — 35% methanol or acetonitrile / 0.1% TFA in water insulin — 35% methanol or acetonitrile / 0.1% TFA in water BSA — 50% acetonitrile / 0.1% TFA in water | | | |
| Flow Rate: | 2mL/min | | | |
| Det.: | UV, 280, 302, or 291nm | | | |
| Inj.: | 5mg/mL of test molecule in the following solution: cephalosporin C in water (pH 2.5); vancomycin or insulin in 0.1% TFA / water; BSA in 0.05M Tris-HCl (pH 8.0) | | | |

Mass recovery of each molecule was >95%.

Styrenic-based Amberchrom CG-161, CG-300 and CG-1000 resins are more retentive for small aromatic molecules than are silica-based reversed phase packings. Low molecular weight pharmaceuticals, such as antibiotics, are best purified on high capacity Amberchrom CG-161 resin. In contrast, retention on methacrylic ester Amberchrom CG-71 is similar to retention on silica-based reversed phase packings. Amberchrom CG-71 is recommended for amino acids, peptides, and small proteins.

Amberchrom resins allow high throughput of process streams, since the pressure drop for a column bed 29cm deep is typically less than 1kg/cm² (15psi). The mechanically stable particles accept flow rates greater than 1500cm/hour with a variety of mobile phases. Pressures up to 6kg/cm² (90psi) do not compress the Amberchrom resins.

Amberchrom resins can operate effectively and with excellent capacity at high velocities. As shown in Table 5, saturation capacity and mass recovery of bovine serum albumin (BSA) remains stable across a 10-fold range in loading flow rate on Amberchrom CG-1000. BSA capacity to the 1% leakage point remains almost constant from 153 to 768cm/hr. Also, when tested with various mobile phases at increased linear velocities, Amberchrom resins demonstrate low back pressures and good mechanical stability (Figure C).

Table 5. Capacity of Amberchrom CG-1000s Resin for BSA at Various Flow Rates

| Loading Flow Rate (cm/hr) | 153 | 307 | 768 | 1539 |
|-----------------------------|---|-----|-----|------|
| Capacity (mg/mL) | 35 | 33 | 28 | 20 |
| Saturation Capacity (mg/mL) | 37 | 35 | 34 | 33 |
| Mass Recovered (%) | 99 | 100 | 90 | 90 |
| Column: | 10mm x 6.3cm | | | |
| Elution: | 0.1% TFA / 45% ACN at loading flow rate | | | |
| Det.: | UV, 280nm | | | |
| Inj.: | 2.5mg/mL BSA in 0.05M Tris-HCl, pH 8.0 | | | |

Figure C. Pressure/Flow Curves for Amberchrom Resins

Column: 22mm ID x 30cm

m grades (50-100µm):

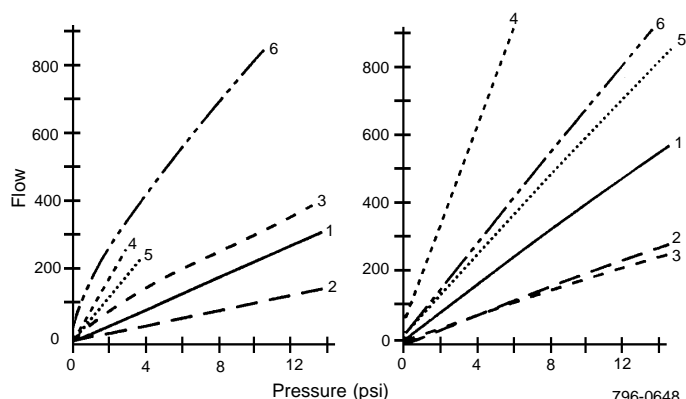
1. ACN
2. 45:55 ACN / H₂O
3. Water

c grades (80-160µm):

4. ACN
5. 45:55 ACN / H₂O
6. Water

Amberchrom CG-161m, CG-161c

Amberchrom CG-71m, CG-71c



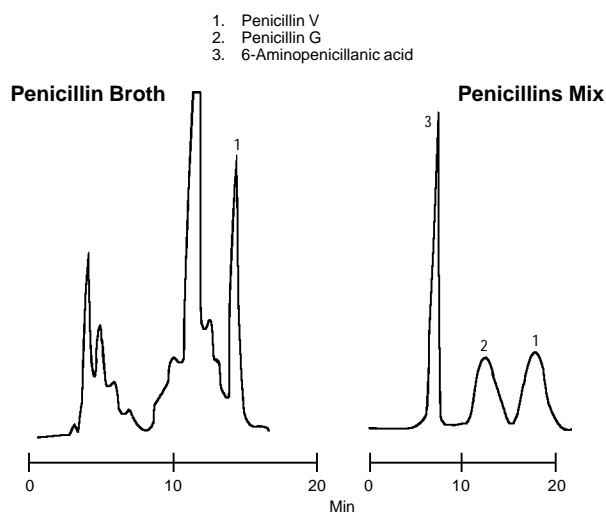
Due to its average pore diameter of 150Å, CG-161 resin has a very low uptake capacity for BSA and insulin compared to CG-300 and CG-1000. However, this low capacity does not mean that CG-161 resin cannot be used to purify polypeptides. At flow rates above 200cm/hr, large polypeptides will be kinetically excluded from a polypeptide feed stream. This means that small hydrophobic impurities will be removed and the polypeptide will be purified and recovered without dilution.

Representative Applications

Production-scale quantities of pharmaceuticals, peptides, and small proteins can be purified by selective desorption on the Amberchrom resins. For example, the selectivity for antibiotics is shown in the separation of penicillin V from its fermentation broth and from closely related derivatives (Figure D).

Figure D. Purify Penicillin with Amberchrom CG-161

Packing: **Amberchrom CG-161m**
 Cat. No.: **10369**
 Column: 10mm ID x 6.3cm
 Mobile Phase: 0.05M phosphate buffer, pH 8.0 (A - 10 min; B - 4 min) to 100% methanol in 25 min
 Flow Rate: 1.0mL/min
 Det.: UV, 280nm



For the separation of high molecular weight biomolecules, wide pore (~1000Å) Amberchrom CG-1000 resins exhibit excellent selectivity (Figure E).

The pH stability of Amberchrom resins allows a broad range of mobile phases for selectivity optimization. This leads to increased resolution and flexibility in analyses of polar compounds, such as strongly basic amines. Figure F shows an example of optimized purification through increased pH. An aspartic acid group on the Angiotensin II peptide differentiates it from Angiotensin III. As the pH increases from 2 to 12, this acid group is neutralized and the retention of Angiotensin II decreases. Maximum resolution is observed at pH 12, where Angiotensin II exhibits the lowest overall retention.

Amberchrom resin has been used routinely for the preparation of high purity oligonucleotides. Offering unique selectivity, Amberchrom resin has been very effective in the development of a one-step purification for DMT-ON oligonucleotides. Crude 20mer phosphodiester oligo was loaded on a column of

Figure E. Separation of Proteins on Wide Pore Amberchrom CG-1000s Resin

Packing: **Amberchrom CG-1000s**
 Cat. No.: **13911**
 Column: 10mm ID x 6.3cm
 Mobile Phase: A = 0.1% TFA in H₂O; B = ACN
 70 min linear gradient from 20% B to 60% B
 Flow Rate: 1mL/min
 Det.: UV, 214nm
 Inj.: 20µL containing 1mg/mL each component listed

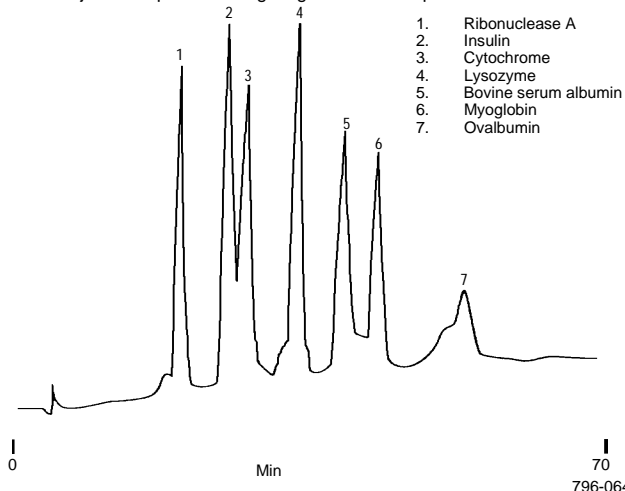
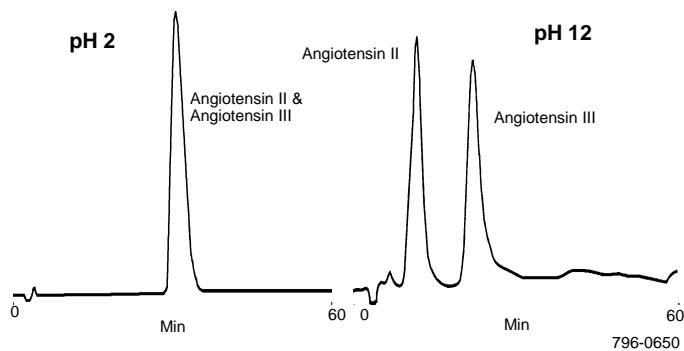


Figure F. Effect of pH on the Separation of Angiotensin Peptides

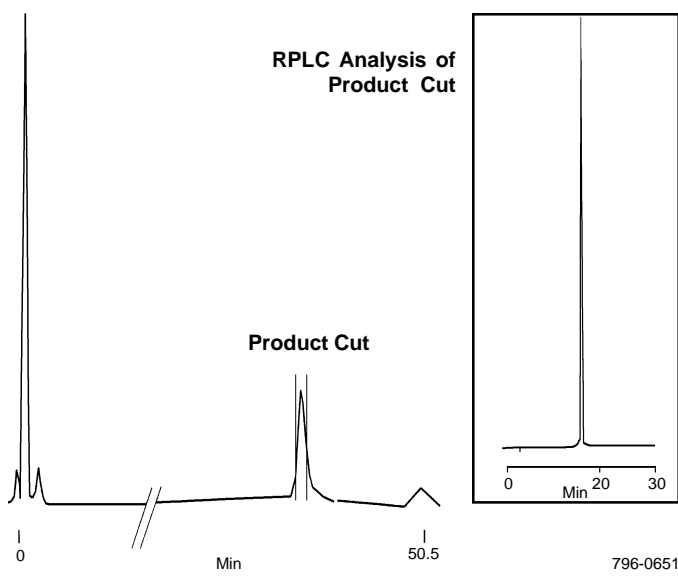
Packing: **Amberchrom CG-300s**
 Cat. No.: **13908**
 Column: 1cm ID x 6.3cm
 Sample: 50µg in 50µL of Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) and Angiotensin III (Arg-Val-Tyr-Ile-His-Pro-Phe)
 Mobile Phase: pH 2 — A = 0.1% TFA / water; B = 60:40 0.1% TFA / ACN:0.1 TFA / H₂O
 pH 12 — A = 10mM NaOH; B = 60:40 ACN:10mM NaOH
 Gradient: 10% B to 30% B in 60 min
 Flow Rate: 1mL/min
 Det.: UV, 214nm, peaks normalized to full scale



Amberchrom CG-300s, then washed to remove impurities. The oligo was detritylated on the column and eluted in an acetonitrile gradient. Fractions eluting in a specific region of the product peak — between half way up the leading edge and half way down the trailing edge — were pooled. Figure G shows the product fractions to be 100% pure by RPLC.

Amberchrom polymeric packings have excellent chromatographic selectivity that enables purification across a broad range of complex mixtures. For example, in Figure H, the capability of Amberchrom resins to distinguish among closely related species is illustrated by their selectivity for parabens.

Figure G. Amberchrom CG-300s Column Purification of DMT-ON Oligonucleotide



Conclusion

Excellent physical, chemical, and thermal stability make Amberchrom resins well suited to process-scale reversed phase separations and purifications of pharmaceuticals, peptides, small proteins, and other small molecules.

Ordering Information:

Amberchrom Resins

100mL slurry

| Resin | Dry Particle Diameter | Cat. No. |
|----------|-----------------------|----------|
| CG-71m | 50-100µm | 10367 |
| CG-71c | 80-160µm | 10366 |
| CG-161m | 50-100µm | 10369 |
| CG-161c | 80-160µm | 10370-U |
| CG-300s | 20-50µm | 13908 |
| CG-300m | 50-100µm | 13909-U |
| CG-300c | 80-160µm | 13910-U |
| CG-1000s | 20-50µm | 13911 |

Trademarks

Amberchrom, Amberlite and XAD are registered trademarks of Rohm & Haas Co.

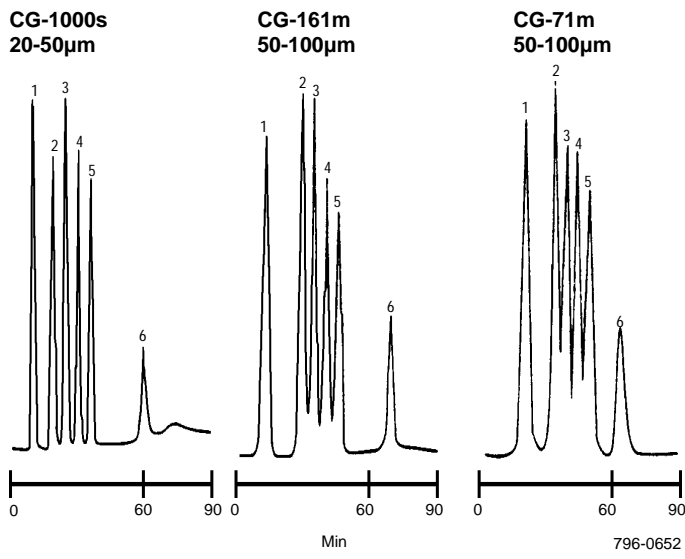
Acknowledgement

Figures A - H used courtesy of TosohHaas, Montgomeryville, PA 18936 USA.

Figure H. Selectivity of Amberchrom Resins

Column: 10mm ID x 6.3cm
 Mobile Phase: A = 0.1% TFA in H₂O; B = ACN
 4 min 30% B, then 64 min linear gradient to 100% B
 Flow Rate: 0.5mL/min
 Det.: UV, 280nm
 Inj.: 100µL containing 100µg (unless otherwise noted) each component

1. *p*-Hydroxybenzoic acid
2. Methyl *p*-hydroxybenzoate
3. Ethyl *p*-hydroxybenzoate
4. Propyl *p*-hydroxybenzoate
5. Butyl *p*-hydroxybenzoate
6. Phenyl salicylate (500µg)



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