About OMICS Group

- OMICS Group is an amalgamation of Open Access Publications and worldwide international science conferences and events. Established in the year 2007 with the sole aim of making the information on Sciences and technology 'Open Access', OMICS Group publishes 500 online open access scholarly journals in all aspects of Science, Engineering, Management and Technology journals. OMICS Group has been instrumental in taking the knowledge on Science & technology to the doorsteps of ordinary men and women. Research Scholars, Students, Libraries, Educational Institutions, Research centers and the industry are main stakeholders that benefitted greatly from this knowledge dissemination. OMICS Group also organizes 500 International conferences annually across the globe, where knowledge transfer takes place through debates, round table discussions, poster presentations, workshops, symposia and exhibitions.

OMICS International Conferences

OMICS International is a pioneer and leading science event organizer, which publishes around 500 open access journals and conducts over 500 Medical, Clinical, Engineering, Life Sciences, Pharma scientific conferences all over the globe annually with the support of more than 1000 scientific associations and 30,000 editorial board members and 3.5 million followers to its credit.

OMICS Group has organized 500 conferences, workshops and national symposiums across the major cities including San Francisco, Las Vegas, San Antonio, Omaha, Orlando, Raleigh, Santa Clara, Chicago, Philadelphia, Baltimore, United Kingdom, Valencia, Dubai, Beijing, Hyderabad, Bengaluru and Mumbai.

APPLICATION OF HPLC TECHNIQUES TO BIOLOGICAL PRODUCTS

HEMANT N. JOSHI, Ph.D., MBA

TARA INNOVATIONS LLC

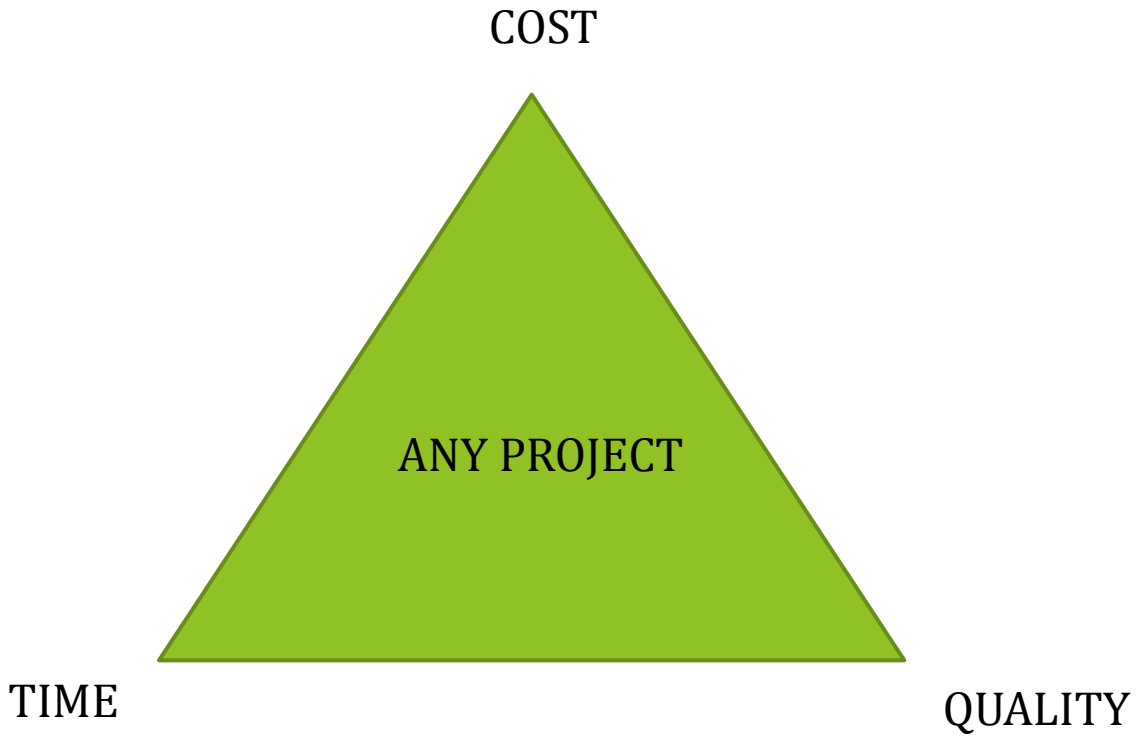
8/10/2015

About Tara Innovations

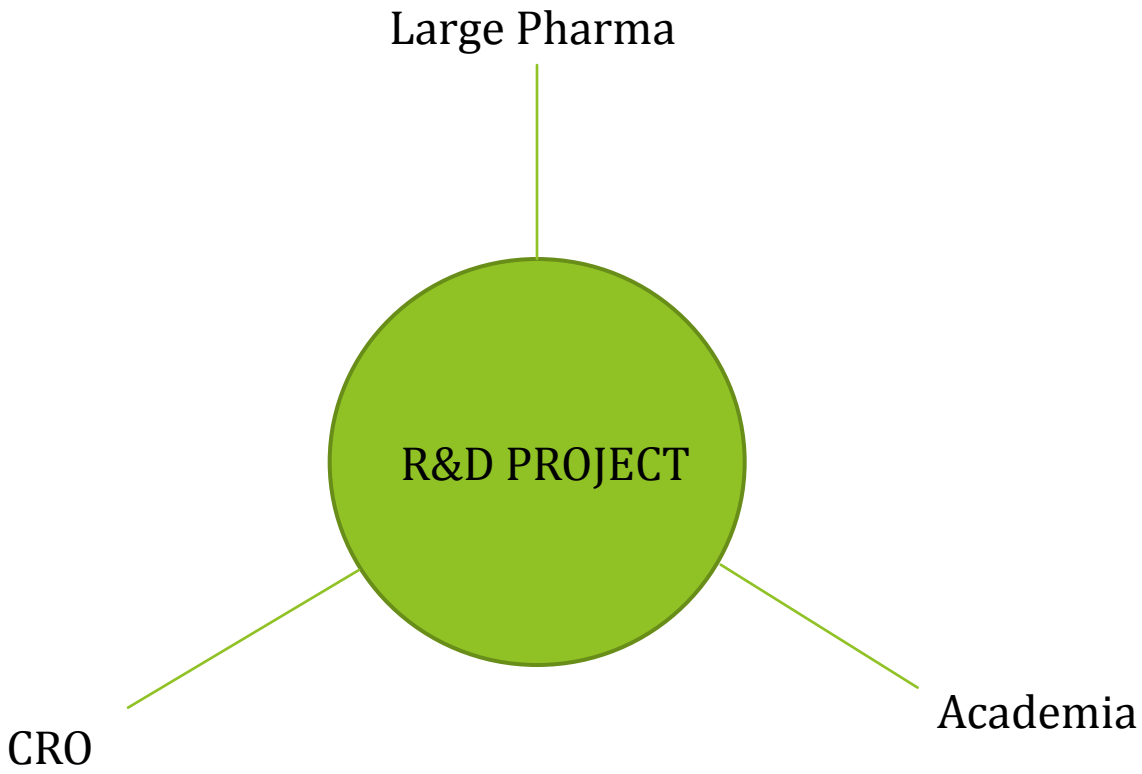
- Tara Innovations LLC is a pharmaceutical CRO, strategy consultant, drug development service provider, and regulatory affairs documentation expert
- Our strength lies in our capability to solve difficult technical and strategic problems
- We specialize in the characterization of small and large molecules, i.e., preformulation activities, development of analytical methods and development of formulations/processes for various types of products.

Mission Statement: To develop products and delivery systems in innovative ways using science and technology in pharmaceutical and nutraceutical fields. We commit to high ethical business standards, meet the changing needs of consumers, and produce intellectual property for the business.

DISCLAIMERS



DISCLAIMERS



Presentation Contents

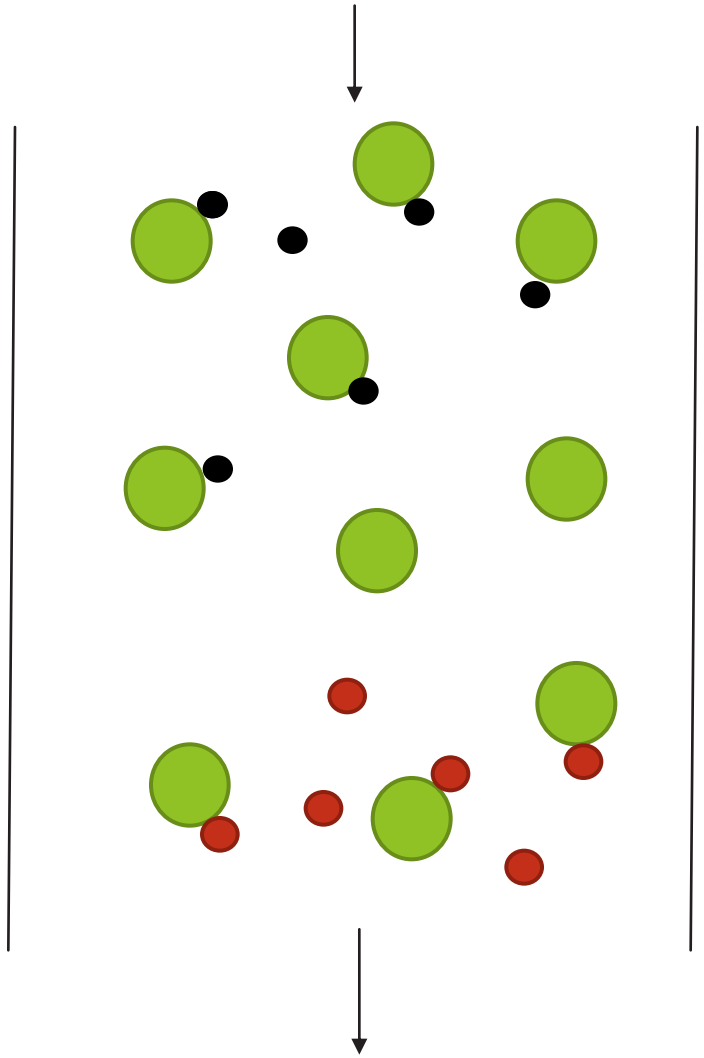
1. **Background on HPLC**
2. **Analysis of a Polypeptide**
3. **Analysis of an Antibody with Size Exclusion Chromatography**
4. **Analysis of an Antibody with Hydrophobic Interaction Chromatography**
5. **Analysis of an Antibody with Ion Exchange Chromatography**
6. **Conclusion**

Background on HPLC

- Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary, while the other (mobile phase) moves in a definite direction.



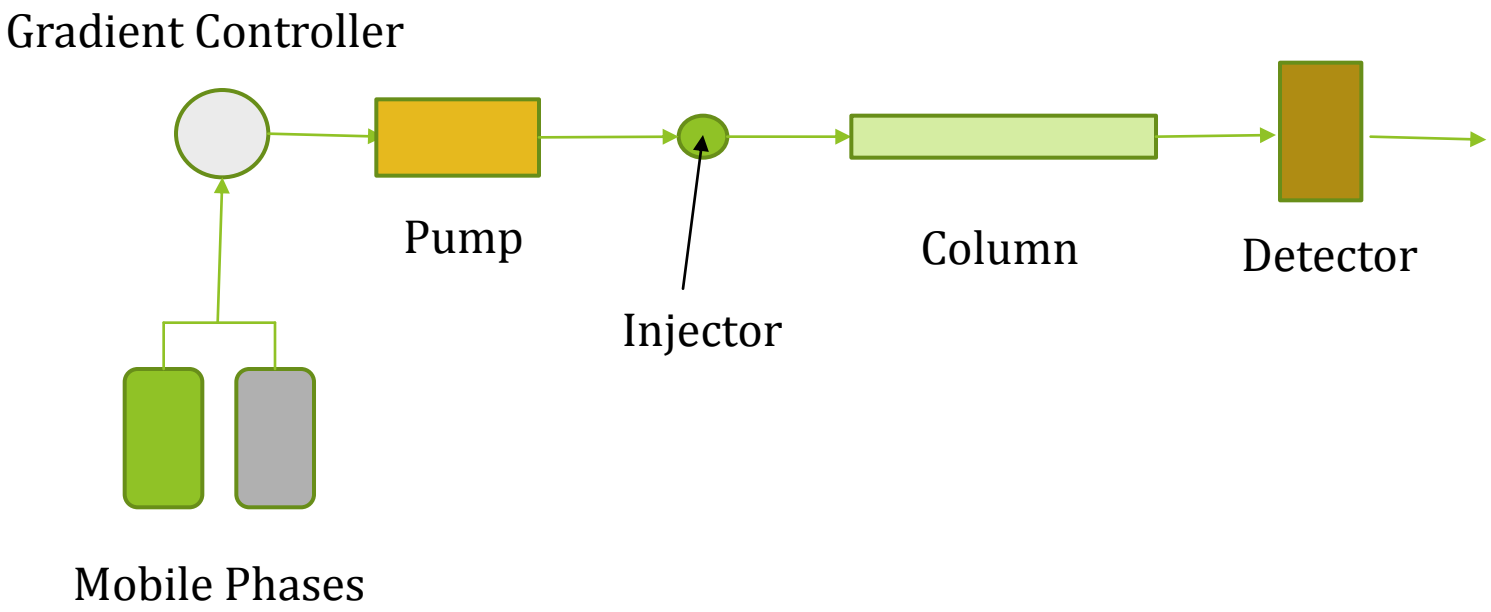
Background on HPLC



Bonded Phases
C2, C8, C18, CN

Background on HPLC

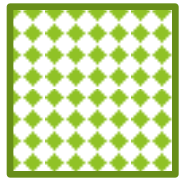
HPLC Instrumentation Layout:



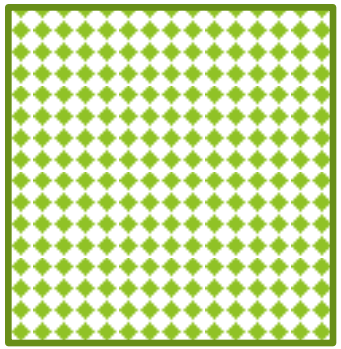
Small vs. Large Drug Molecules



9 diamonds = 900 MW



100 diamonds = 10,000 MW

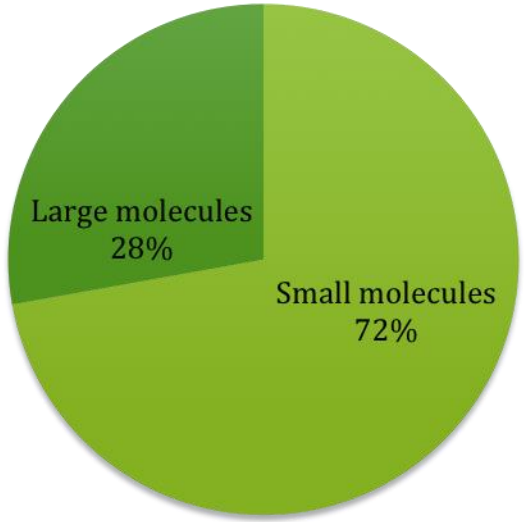


400 diamonds = 40,000 MW

1 kg = 6.02213665168E+23 kilodalton = 1000 grams
100 kilodalton = 1.661E-1 grams

Small vs. Large Molecules

Large molecules vs Small molecules – NDAs in 2014

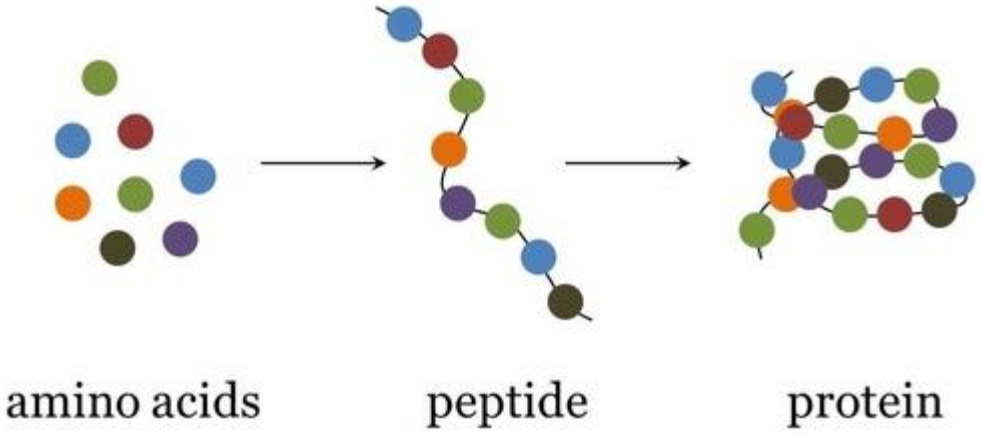


Ref. : H. Sant, R. Kelkar and H. Joshi, Horizon Lines – A yearly Review of NDAs in 2014 Pharmaceutical Outsourcing : 52, 54, 55 (July-August, 2015)

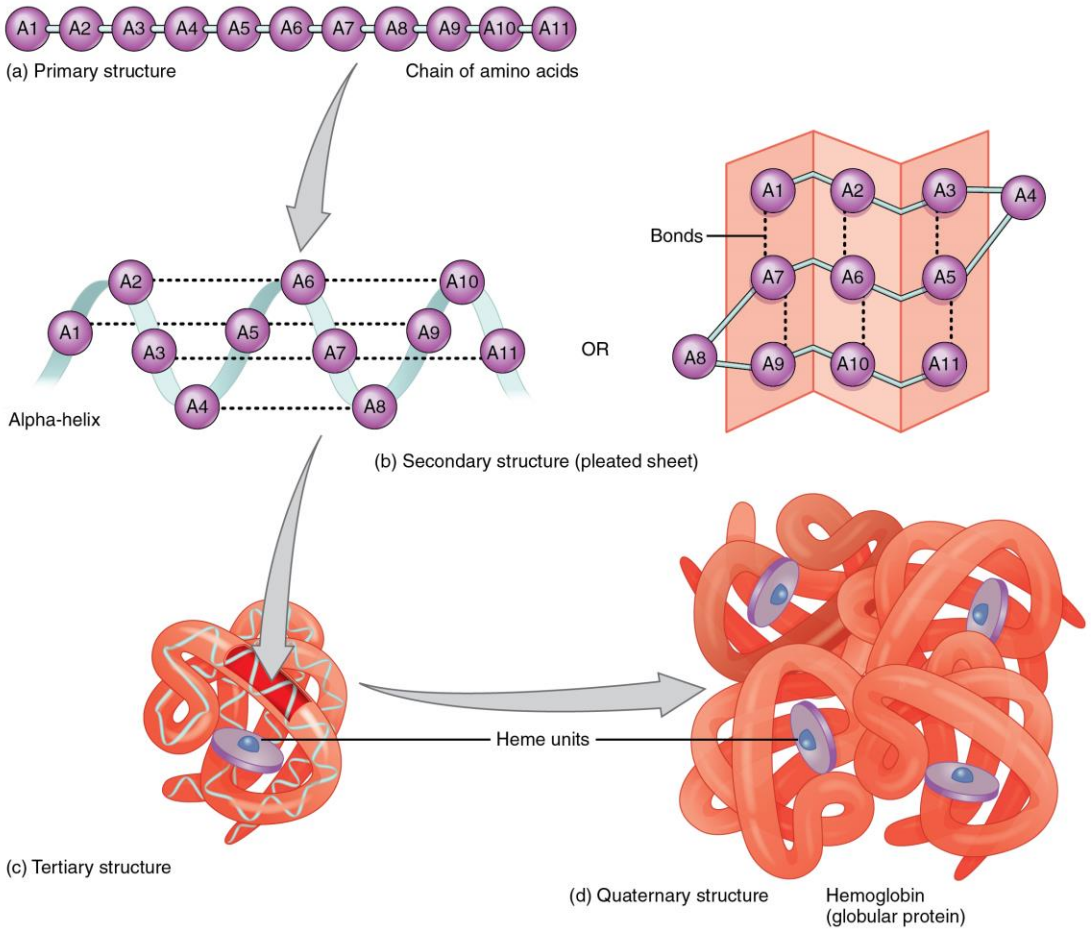
Biological Products

- A biological product is a “virus, therapeutic serum, toxin, antitoxin, vaccine, blood, blood component or derivative, allergenic product, or analogous product, applicable to the prevention, treatment or cure of a disease or condition of human beings”.
- Examples of therapeutic biological entities are – cytokines, growth factors, enzymes, monoclonal antibodies, Fab fragments etc.

HPLC Analysis of a Polypeptide

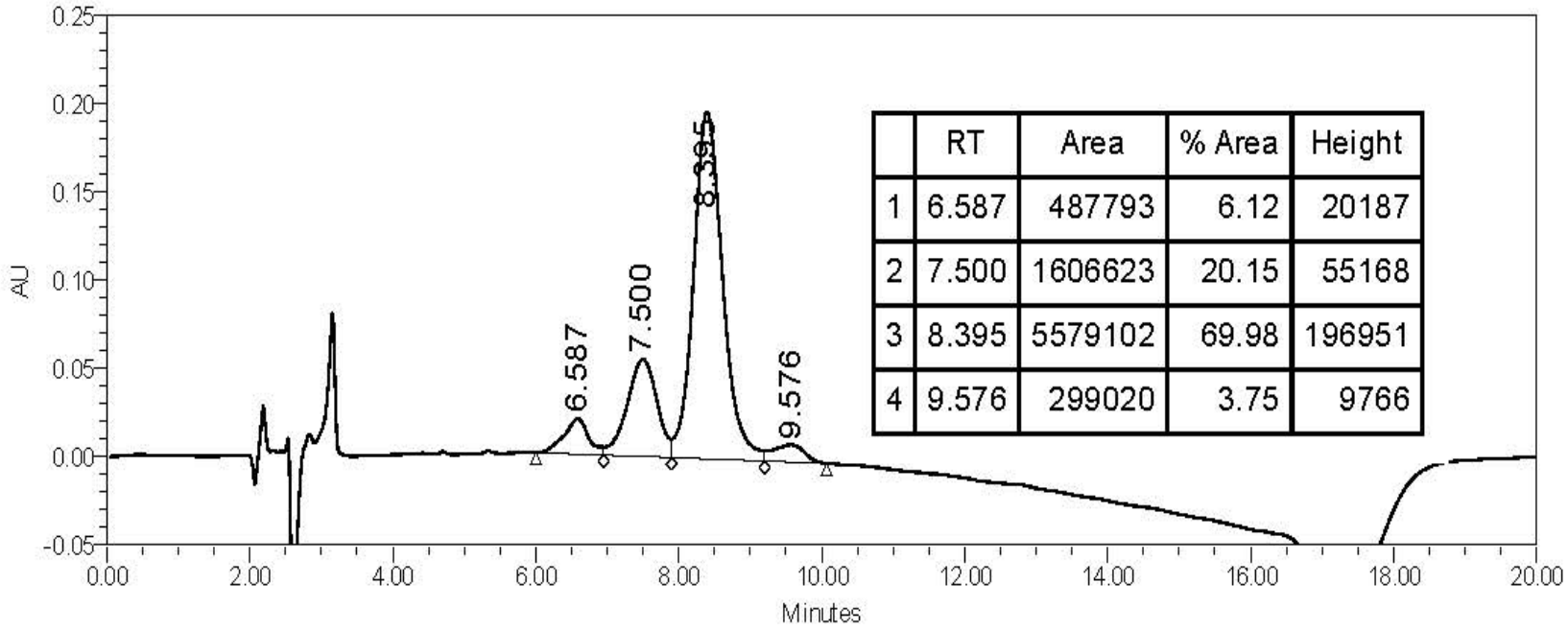


HPLC Analysis of a Polypeptide



HPLC Analysis of a Polypeptide

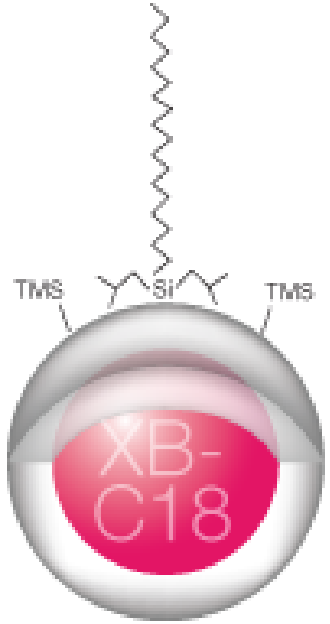
Sample chromatogram of a polypeptide using normal C18 column



	RT	Area	% Area	Height
1	6.587	487793	6.12	20187
2	7.500	1606623	20.15	55168
3	8.395	5579102	69.98	196951
4	9.576	299020	3.75	9766

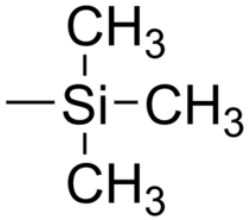
HPLC Analysis of a Polypeptide

- Core Shell technology – Porous coating on a solid core.
- The reduced diffusion path improves efficiency.



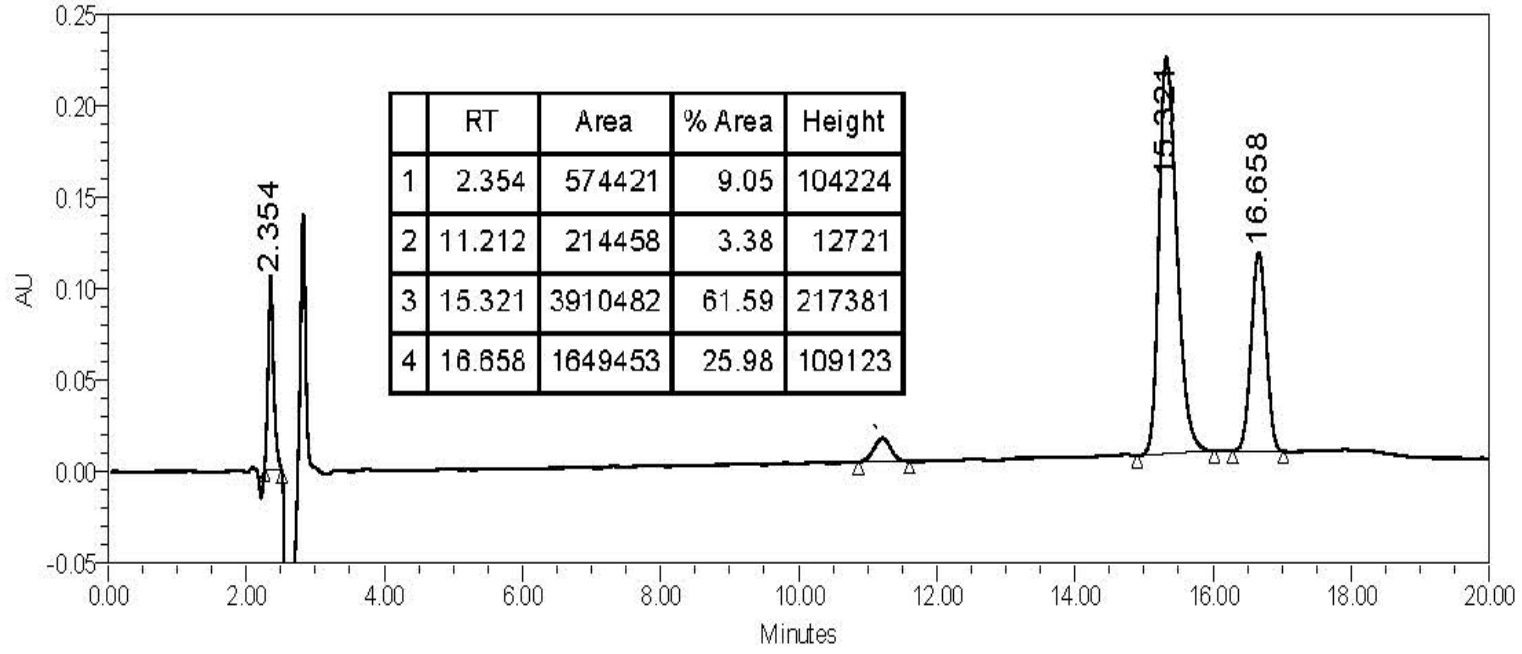
- C18 with iso-butyl octadecylsilyl side chains, TMS end-capping, and core silica shell
- C4 Column – For very hydrophobic proteins
- C8 Column – For hydrophobic proteins
- C18 Column – For hydrophilic proteins

- TMS or Trimethyl Silyl



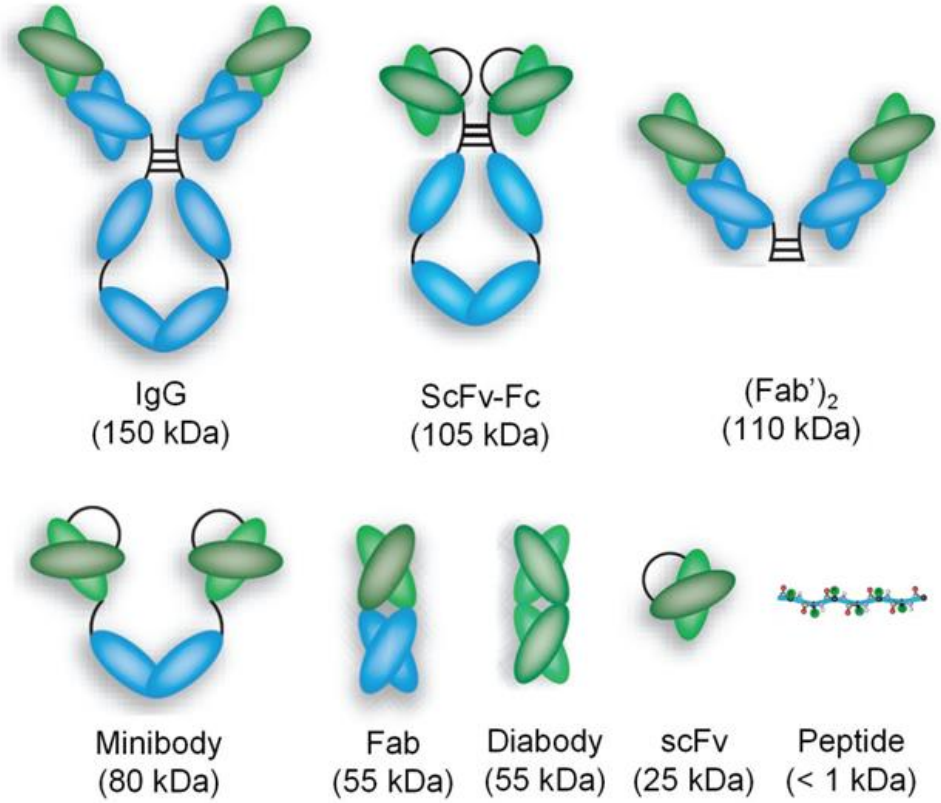
HPLC Analysis of a Polypeptide

Sample chromatogram of a polypeptide using a Kinetex XB C18 column

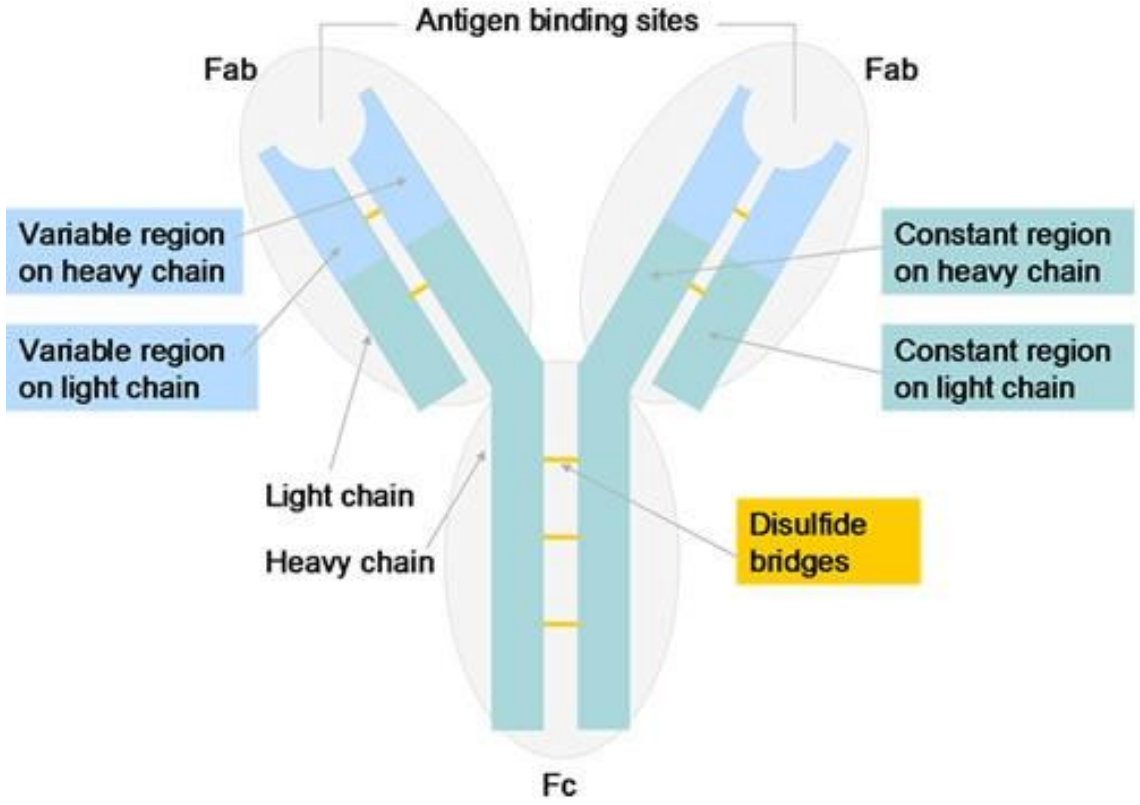


	RT	Area	% Area	Height
1	2.354	574421	9.05	104224
2	11.212	214458	3.38	12721
3	15.321	3910482	61.59	217381
4	16.658	1649453	25.98	109123

Antibody Structure



Antibody Structure



Antibody Structure

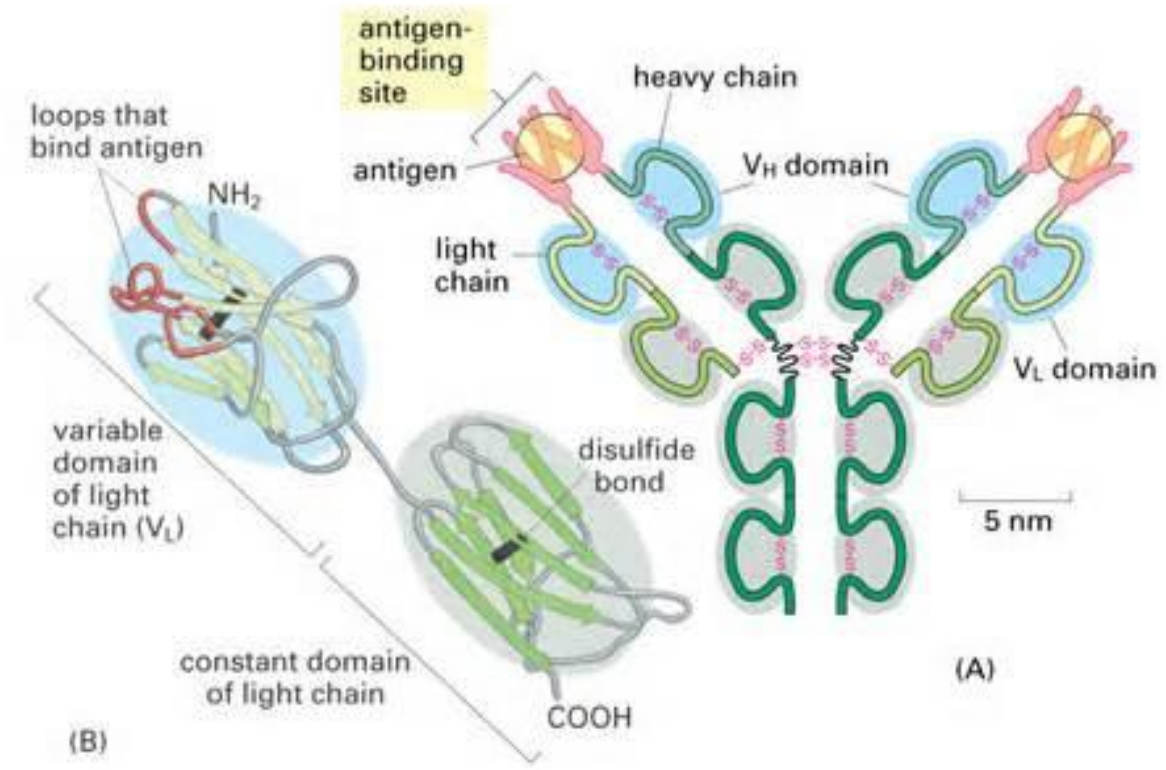
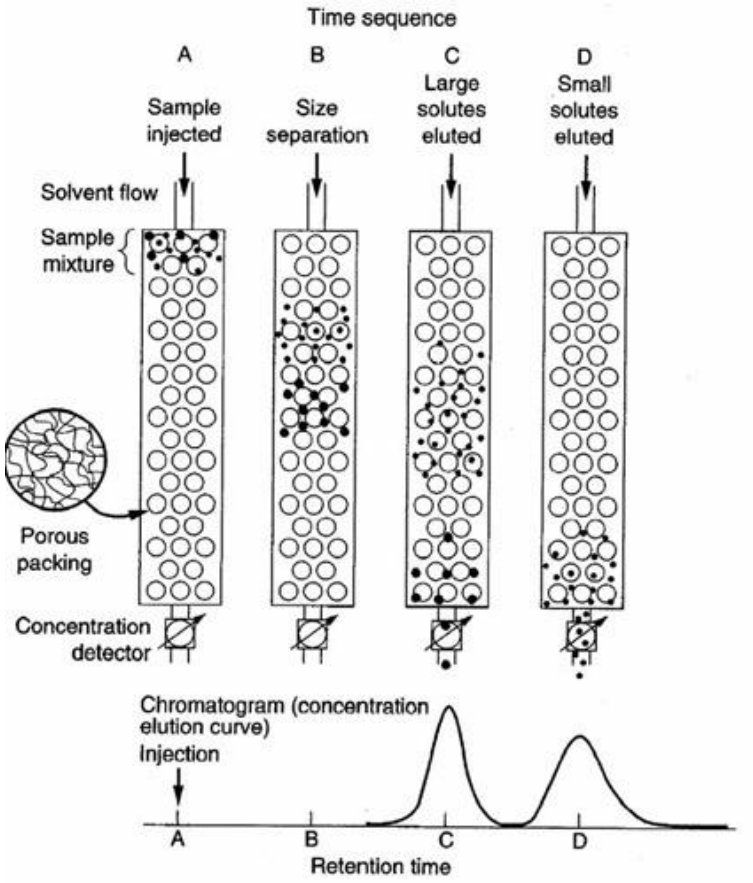


Figure 4-32 Essential Cell Biology, 2/e. (© 2004 Garland Science)

Size Exclusion Chromatography

- SEC is a chromatographic method in which the analyte molecules are separated by their size.
- One key requirement is – the analytes do not interact with the surface of the stationary phases. Small molecules penetrate every region/pore and elute late.
- There are various measures for the sizes of macromolecules and hydrodynamic volumes are commonly used.
- The SEC columns are segregated based on the MW range they can separate.
- Increasing the column length enhances the resolution.
- Increasing the column diameter increases the capacity of the column.

Size Exclusion Chromatography



Size Exclusion Chromatography

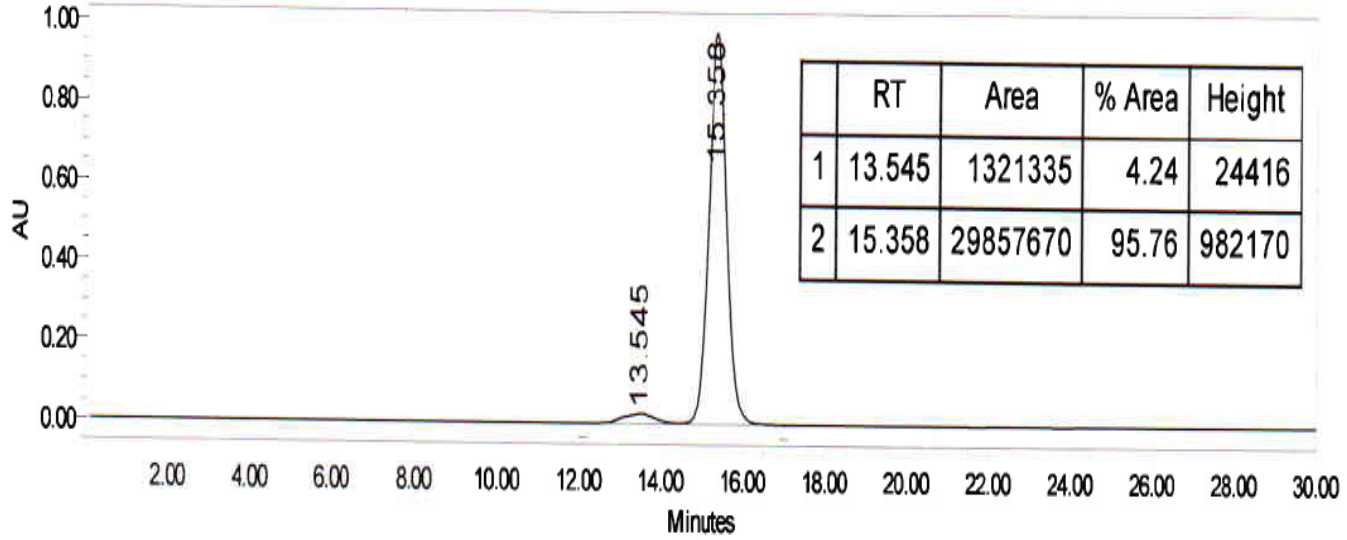
- Details on the SEC Method Development:
  - HPLC Pump – Waters Alliance 2695
  - HPLC Detector – Waters 2487
  - Antibody MW – 100 to 150 kilodalton
  - Concentration – 5 mg/mL
  - Diluent – 50 mM phosphate buffer, pH 7.2, with 220 mM sodium chloride

Size Exclusion Chromatography

- Column: YMC-pack-Diol 200, 300 x 8 mm, S 5 µm, 20 nm, catalog no. DL20S05-3008WT
- Mobile Phase: 50 mM phosphate, 150 mM NaCl, pH 7.2 at room temperature
- Flow Rate: 0.5 ml/min
- Run Time: 30 min
- Detection: 220 nm
- Injection: 20 µL at 1 mg/mL (dilute the samples with DI water)
- Column Storage: 10 mM phosphate pH 7.0 with 0.05% Sodium Azide

Size Exclusion Chromatography

Sample Chromatogram



	RT	Area	% Area	Height
1	13.545	1321335	4.24	24416
2	15.358	29857670	95.76	982170

Size Exclusion Chromatography

Effect of Flow Rate

Flow Rate, mL/min	Aggregate RT, min	% Aggregate	Monomer RT, min	% Monomer
0.4	16.95	4.2	17.0	95.8
0.5	13.55	4.5	13.6	95.5
0.6	11.28	4.2	11.3	95.8

Size Exclusion Chromatography

Effect of Salt Concentration

Salt mM	% Aggregate	% Monomer
125	4.1	95.9
150	4.5	95.5
175	4.4	95.6

Size Exclusion Chromatography

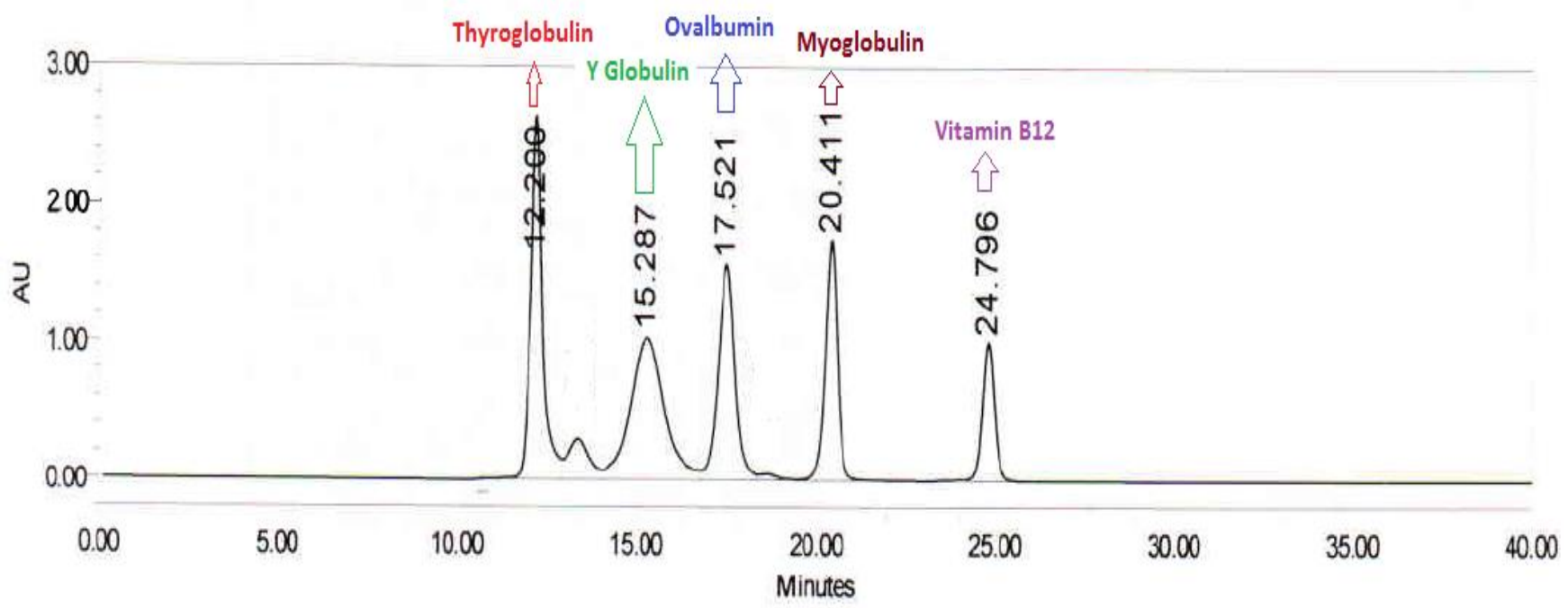
Effect of pH of Mobile Phase

Mobile Phase pH	% Aggregate	% Monomer
7.0	3.9	96.1
7.2	4.5	95.5
7.4	4.3	95.7

Size Exclusion Chromatography

- A gel standard mixture was purchased from Bio-rad. It contained the following:
  - A = Protein Aggregate
  - B = Thyroglobulin, MW = 670,000
  - C = Gamma-globulin, MW = 158,000
  - D = Ovalbumin, MW = 44,000
  - E = Myoglobulin, MW = 17,000
  - F = Vitamin B12, MW = 1,350

Size Exclusion Chromatography



Size Exclusion Chromatography

Effect of Basic pH (pH 10.32) on the Stability of Antibody AB

Incubation Time, minutes	Peak Area
80	32385549
160	32383764
240	32538806
560	32658370

Size Exclusion Chromatography

Effect of Acidic pH (pH of 1.23) on the Stability of Antibody AB

Incubation Time, mins	Peak Area	% Peak Area
40	2756396	92.0
120	1382033	55.0
200	1312084	27.3
520	1548169	27.6

Size Exclusion Chromatography

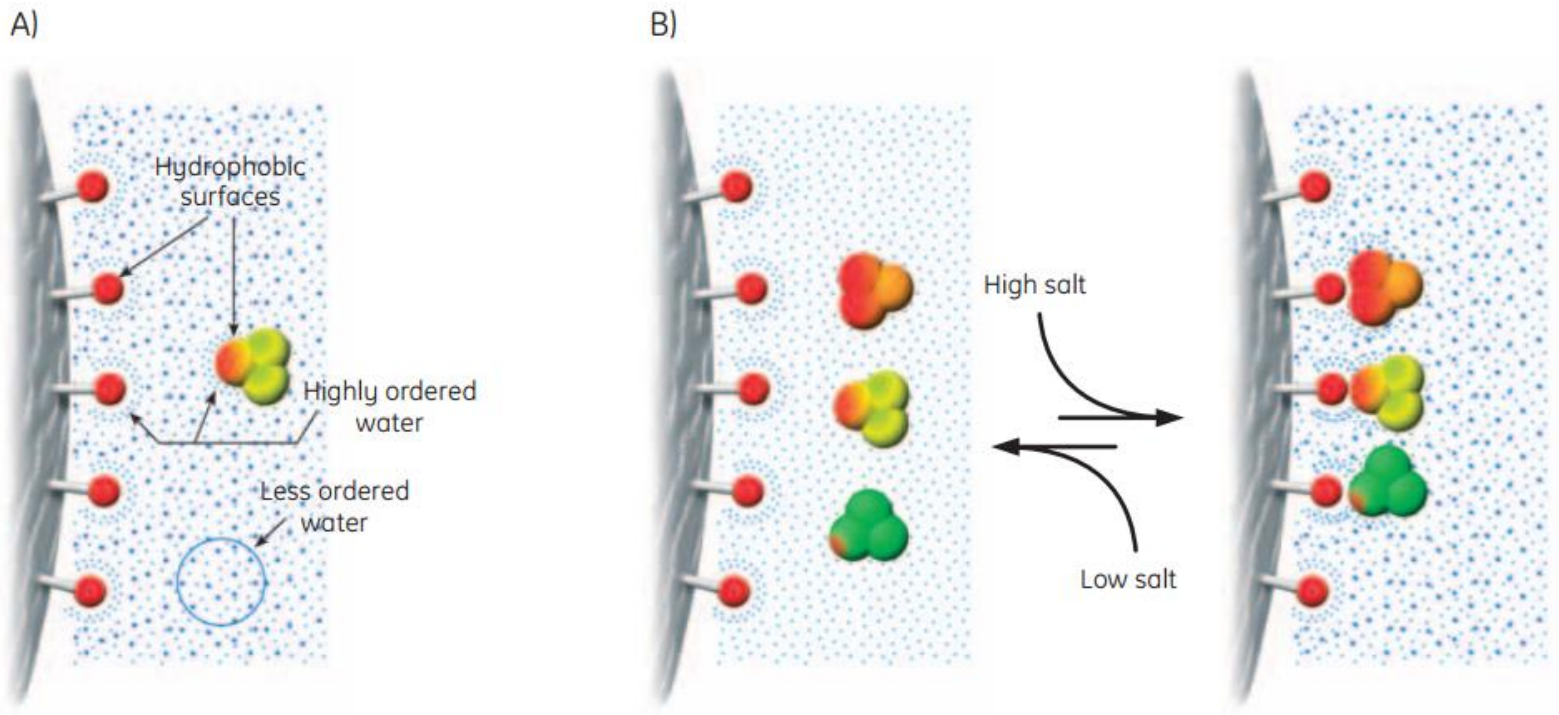
- **Conclusion:**
  - The SEC method for Antibody AB was satisfactory
  - The method was stability-indicating

Hydrophobic Interaction Chromatography

- Hydrophobic Interaction chromatography (HIC) is a chromatographic technique that separates components based on the interaction of hydrophobic groups of the molecule, with hydrophobic ligands on media.

- Used ProPac HIC 10 5 µm, 2.1 x 100 mm, 300 Å column from Thermo Scientific and an aqueous buffered mobile phase.

Hydrophobic Interaction Chromatography



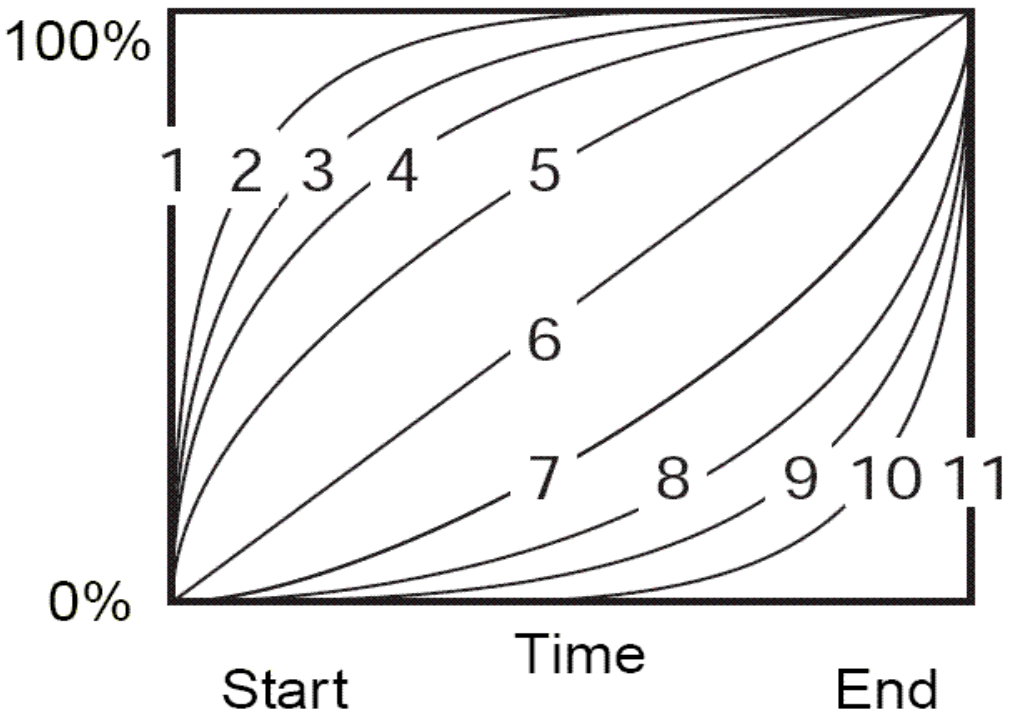
A) Highly ordered water shells surround the hydrophobic surfaces of ligands and proteins. Hydrophobic substances are forced to merge to minimize the total area of such shells (maximize entropy). Salts enhance the hydrophobic interaction. B) The equilibrium of the hydrophobic interaction is controlled predominantly by the salt concentration.

Hydrophobic Interaction Chromatography

- In the beginning, the following variables were attempted to develop a better chromatography:

1. Using water as mobile phase C
2. Changing the slope of gradient by changing the A:B composition at midpoint
3. Changing the time of midpoint - such as 31 or 51 minutes
4. Flow rates (0.5 mL/min or 0.6 mL/min)
5. Increasing the amount of ammonium sulfate. The original mobile phase B contained 400 mM of ammonium sulfate. Attempted to use 500 mM and 750 mM ammonium sulfate.
6. Column temperature – RT, 30° C or 40° C

Hydrophobic Interaction Chromatography



Hydrophobic Interaction Chromatography

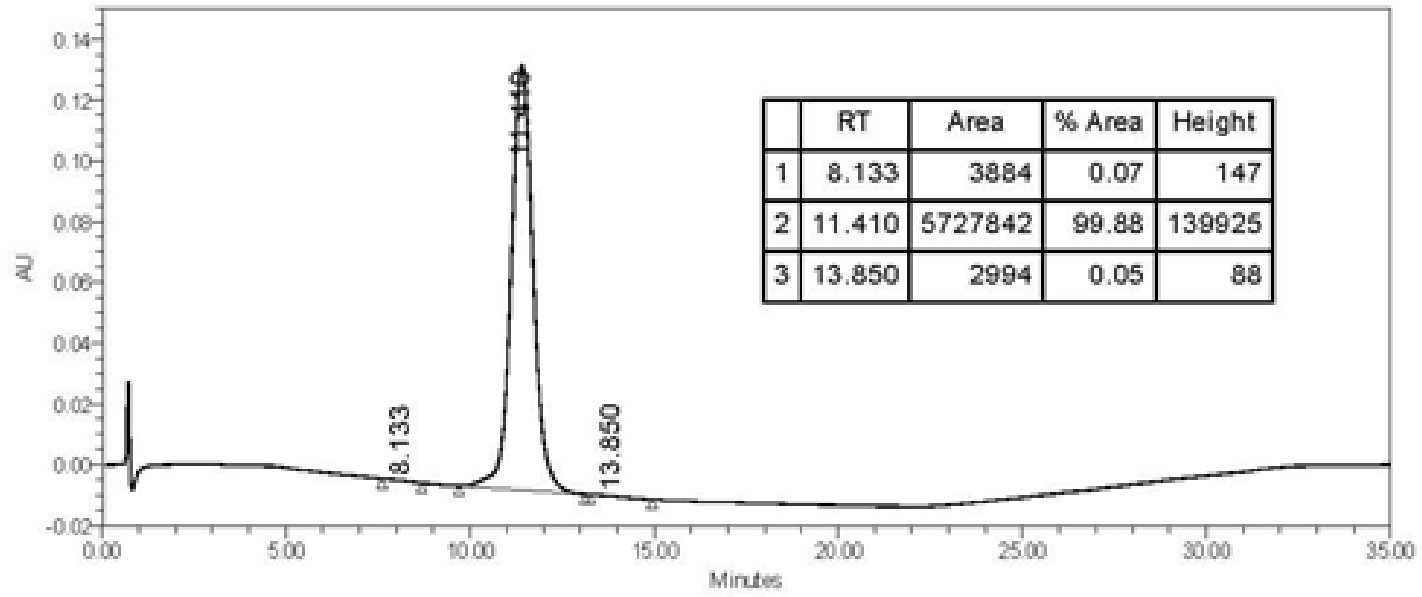
- Column: ProPac HIC 10, 5 µm, 2.1 x 100 mm, 300 Å, Thermo Scientific
- Mobile phase A – 0.37 g of sodium monobasic phosphate + 0.62 g of sodium dibasic phosphate + 250 mg of sodium azide – final volume to 1 L.
- Mobile Phase B – 1.34 g of sodium dibasic phosphate + 132.15 g of Ammonium sulfate + 250 mg of sodium azide – final volume to 1 L.
- Mobile phase C - Acetonitrile
- Wavelength of detection – 280 nm
- Column temperature – 40° C
- Flow rate – 0.5 mL/min

Hydrophobic Interaction Chromatography

Time (min)	Mobile Phase A	Mobile Phase B	Mobile Phase C	Curve
0	2	98	0	6
1	2	98	0	6
10	50	47	3	6
20	60	37	3	6
30	2	98	0	6
35	2	98	0	6

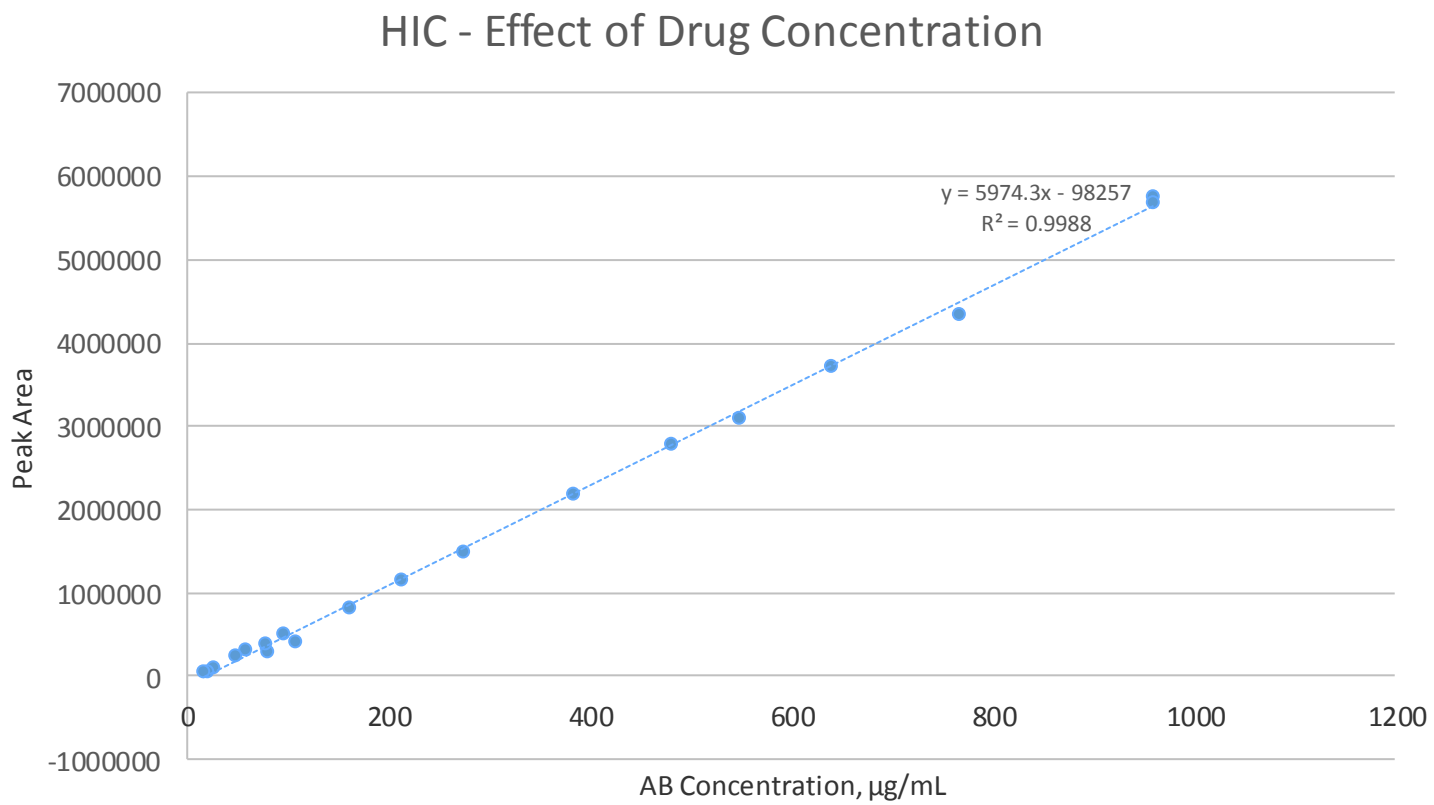
Column Storage: 10 mM Phosphate pH 7.0 with 0.05% Sodium Azide

Hydrophobic Interaction Chromatography



	RT	Area	% Area	Height
1	8.133	3884	0.07	147
2	11.410	5727842	99.88	139925
3	13.850	2994	0.05	88

Hydrophobic Interaction Chromatography



Hydrophobic Interaction Chromatography

Oxidation of Antibody AB with ChloramineT

µg CT/mg drug	% Main Peak
0	100
1.84	86.63
3.68	68.16
5.55	56.54
46.29	53.06
77.78	53.86

Hydrophobic Interaction Chromatography

Stability of Antibody AB in Acid and Base (peak area values)

Time	Effect of HCl	Effect of NaOH
0 time before NaOH or HCl	5,921,995	5,875,475
Immediately after the addition of NaOH or HCl	1,461,114	5,745,100
After 1 day at 25° C	49,315	5,782,210

Ion-Exchange Chromatography

- Proteins, peptides, antibodies and other large molecules have groups with positive or negative charges on the surface.
- There could be a net positive, negative or no charge (PI or isoelectric point) at a specified pH value.
- In an Ion-Exchange Chromatography (IEC), molecules are separated based on their charge.

Ion-Exchange Chromatography

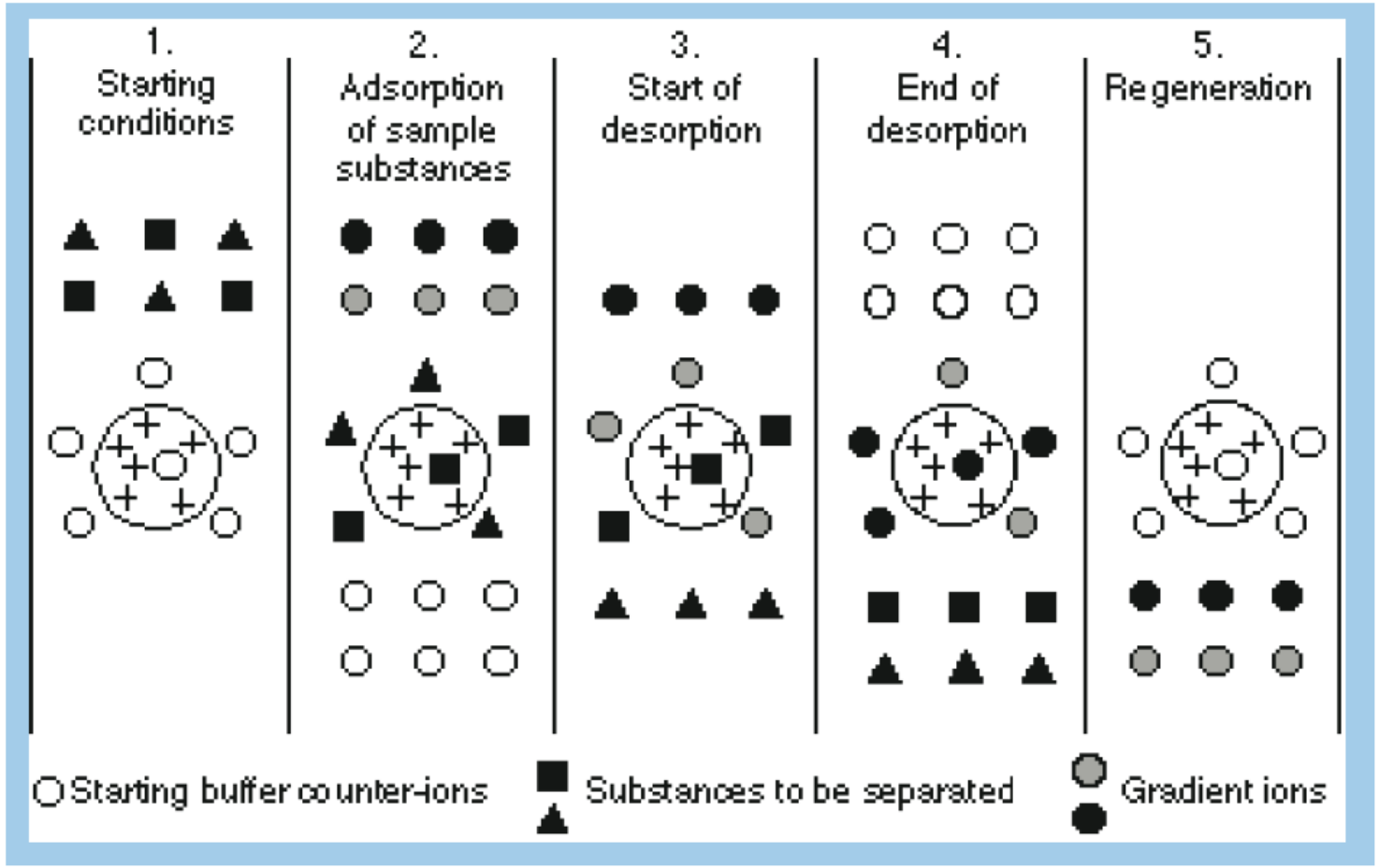
- As a rule, the pH of the mobile phase buffer must be between the pI (isoelectric point, no charge on the protein) or pKa (acid dissociation constant) of the charged molecule and the pKa of the charged group on the solid support (stationary phase).
- Some of the parameters varied were:
  - pH of Mobile phase A
  - pH of Mobile phase B
  - Using Acetonitrile as Mobile phase C
  - Increasing the salt content of Mobile phase B
  - Temperature of the column
  - Variations in the gradient

Ion-Exchange Chromatography

- Dionex ProPac WCX-10 is a weak cation-exchange column, which has carboxylate functional groups.
- The stationary phase is composed of 10 mm non-porous ethylvinyl benzene-divinylbenzene copolymer beads, which are surrounded by a highly hydrophilic neutral polymer.
- The hydrophilic layer prevents unwanted secondary interactions.

- In the cation exchange chromatography, positively charged cations are retained on the negatively charged stationary phase.
- By adjusting the pH or the ionic concentration of the mobile phase, various protein molecules can be separated.

Ion-Exchange Chromatography



Ion-Exchange Chromatography

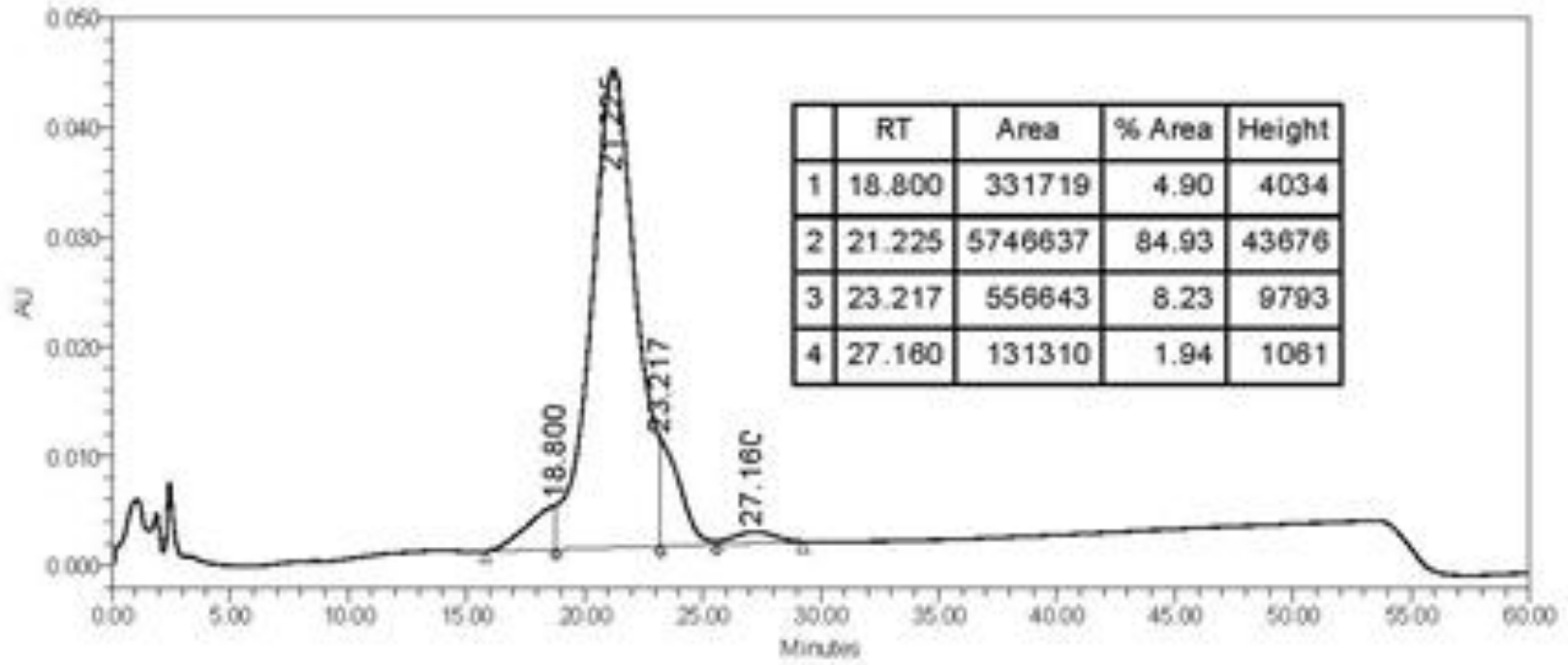
- Column: Thermo Scientific Pro-Pac™ WCX-10 BioLC™, Serial # 024728, Lot # 013-29-070, 4 x 250 mm
- Mobile Phases:
  - A: 10 mM phosphate buffer, pH 5.50 with 0.015% sodium azide. Used Monobasic and dibasic phosphate buffer solutions.
  - B: 10 mM phosphate buffer, pH 7.50 with 0.015% sodium azide and 200 mM sodium chloride. Added 0.1 N HCl to 10 mM dibasic sodium phosphate solution to lower the pH to 7.50.
  - Flow Rate: 0.5 ml/min
  - Run Time: 60 min
  - Wavelength of Detection: 280 nm
- ***A combination of salt and pH gradient was used to analyze BC8 by IEC***

Ion-Exchange Chromatography

Time, min	Mob. Phase A	Mob. Phase B
0	91	9
2	91	9
50	20	80
51	91	9
60	91	9

Ion-Exchange Chromatography

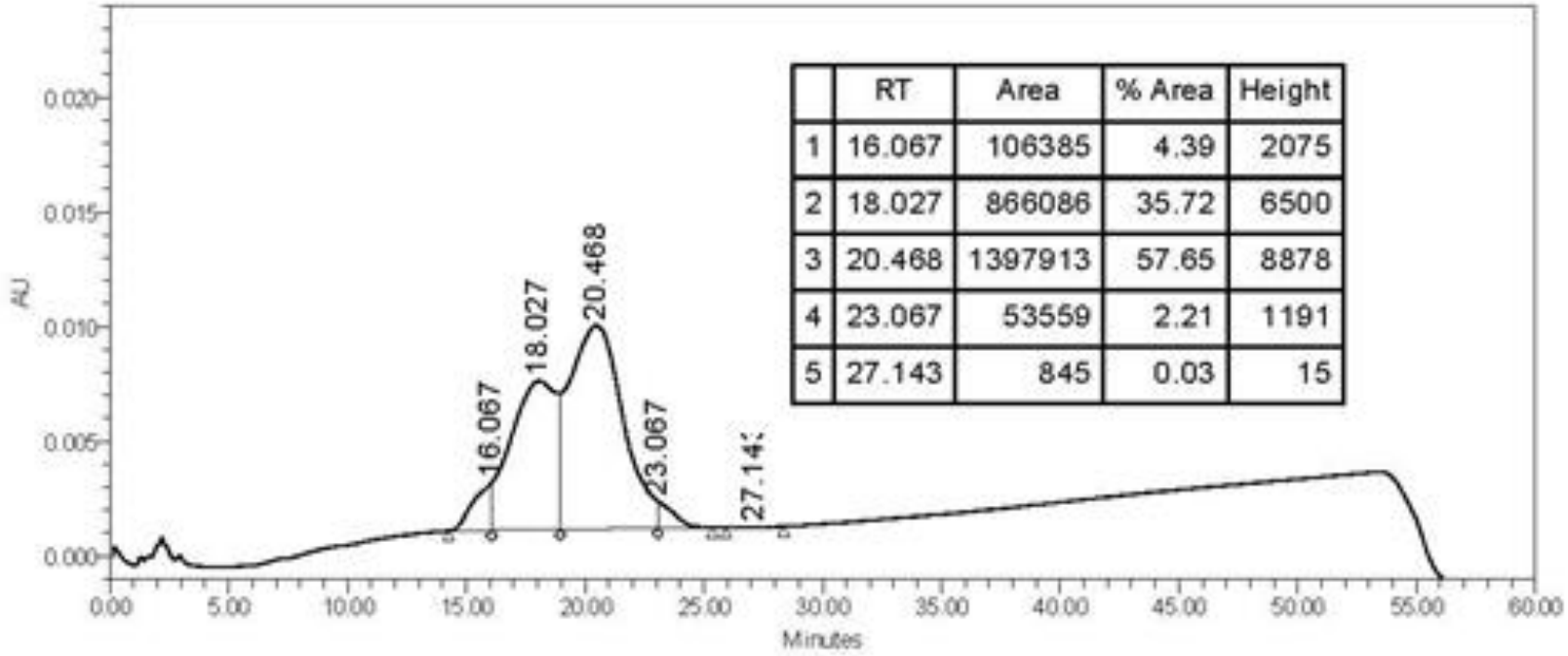
Sample Chromatogram of Antibody AB



	RT	Area	% Area	Height
1	18.800	331719	4.90	4034
2	21.225	5746637	84.93	43676
3	23.217	556643	8.23	9793
4	27.160	131310	1.94	1061

Ion-Exchange Chromatography

Chromatogram of CT-oxidized Antibody AB



	RT	Area	% Area	Height
1	16.067	106385	4.39	2075
2	18.027	866086	35.72	6500
3	20.468	1397913	57.65	8878
4	23.067	53559	2.21	1191
5	27.143	845	0.03	15

Conclusion

- HPLC is a very powerful technique useful for the analysis of small and large molecules.
- Several types of new columns are available in the market and should be tried out during method development.

Thank You!

Dr. Hemant N. Joshi, Ph.D., MBA (founder)
hemantjoshi@tarainnovations.com

Tara Innovations LLC
Lab : (973) 585 7010
Mobile : (973) 998 1565

Let us meet again..

We welcome you all to our future conferences of OMICS International

7th Annual Global Pharma Summit

On

June 20-22, 2016 at New Orleans**, USA**

http://american.pharmaceuticalconferences.com/