ION EXCHANGE CHROMATOGRAPHY (IEC)

APPLICATION

• mixtures of inorganic ions (ion chromatography)

• biomolecules, including amino acids, peptides, proteins, and especially

oligonucleotides

• carbohydrates

• carboxylic acids

• sample preparation

• two-dimensional separation

Values of the retention factor k in IEC for a univalent counter-ion $Y+$ or $Y-$ in cation- or anion-exchange respectively can be derived from the equilibrium of either

Equation log

$$k = a - m\log C$$

- where C is the molar concentration of the counter-ion $Y+$ or $Y-$ in the mobile phase, a is a constant (equal to log k for $C = 1M$), and m is the absolute value of the charge z on the solute molecule X; a and m are constants for a given sample compound, column, salt, buffer, mobile phase pH, and temperature.

Role of the Counter-Ion present in mp

- Mobile phases for IEC usually consist of water, a buffer to control pH, and a salt (or counter-ion) to adjust sample retention (solvent-strength control).

- Different mobile-phase counter-ions are retained more or less strongly by ion exchange, so that a change in the counter-ion can also be used to increase or decrease solvent strength and overall sample retention. Generally, counter-ions with a higher charge will be more effective at reducing sample retention. The relative ability of an ion to bind more strongly, suppress sample retention, and provide smaller values of k increases in the following order

(anion exchange) F^- (larger values of k for solutes) $< OH^- < acetate^- < Cl^- <$

$SCN^- < Br^- < NO_3^- < I^- < oxalate^{-2} < SO_2^{-2} <$

$citrate^{-3}$ (smaller values of k)

(cation exchange) Li^+ (larger values of k for solutes) $< H^+ < Na^+ < NH_4^+ <$

$K^+ < Rb^+ < Cs^+ < Ag^+ < Mg^{+2} < Zn^{2+} < Co^{2+} < Cu^{2+} <$

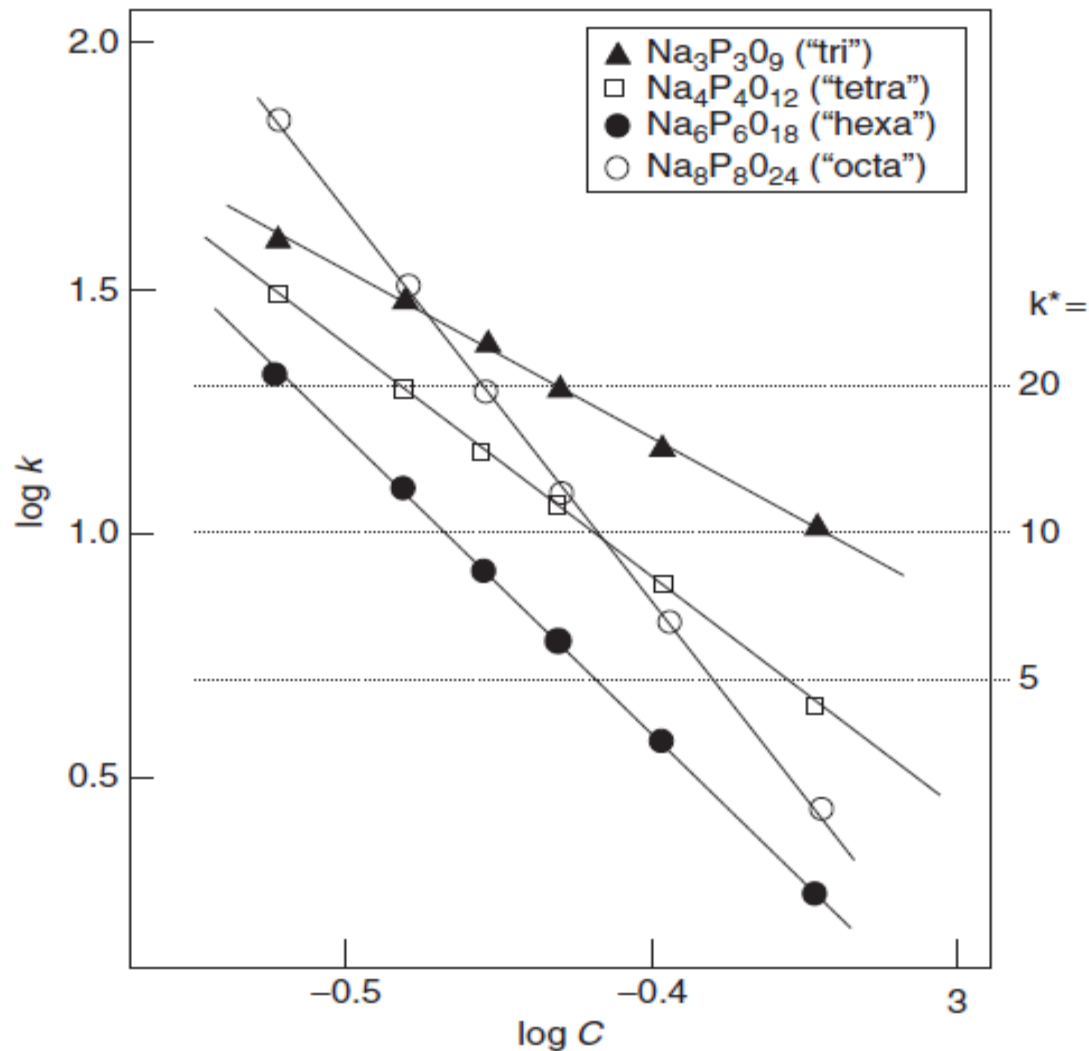$Cd^{2+} < Ni^{2+} < Ca^{2+} < Pb^{2+} < Ba^{2+}$ (smaller values of k)

Figure 7.19 Illustration of the dependence of log k on counter-ion concentration (log C) in isocratic IEC. Sample: four polyphosphates described in figure; conditions: 500 × 4.0-mm TSKgel SAX anion exchange column; aqueous KCl salt solutions (buffered at pH-10.2 with EDTA) as mobile phase; 30°C. Adapted from [64].
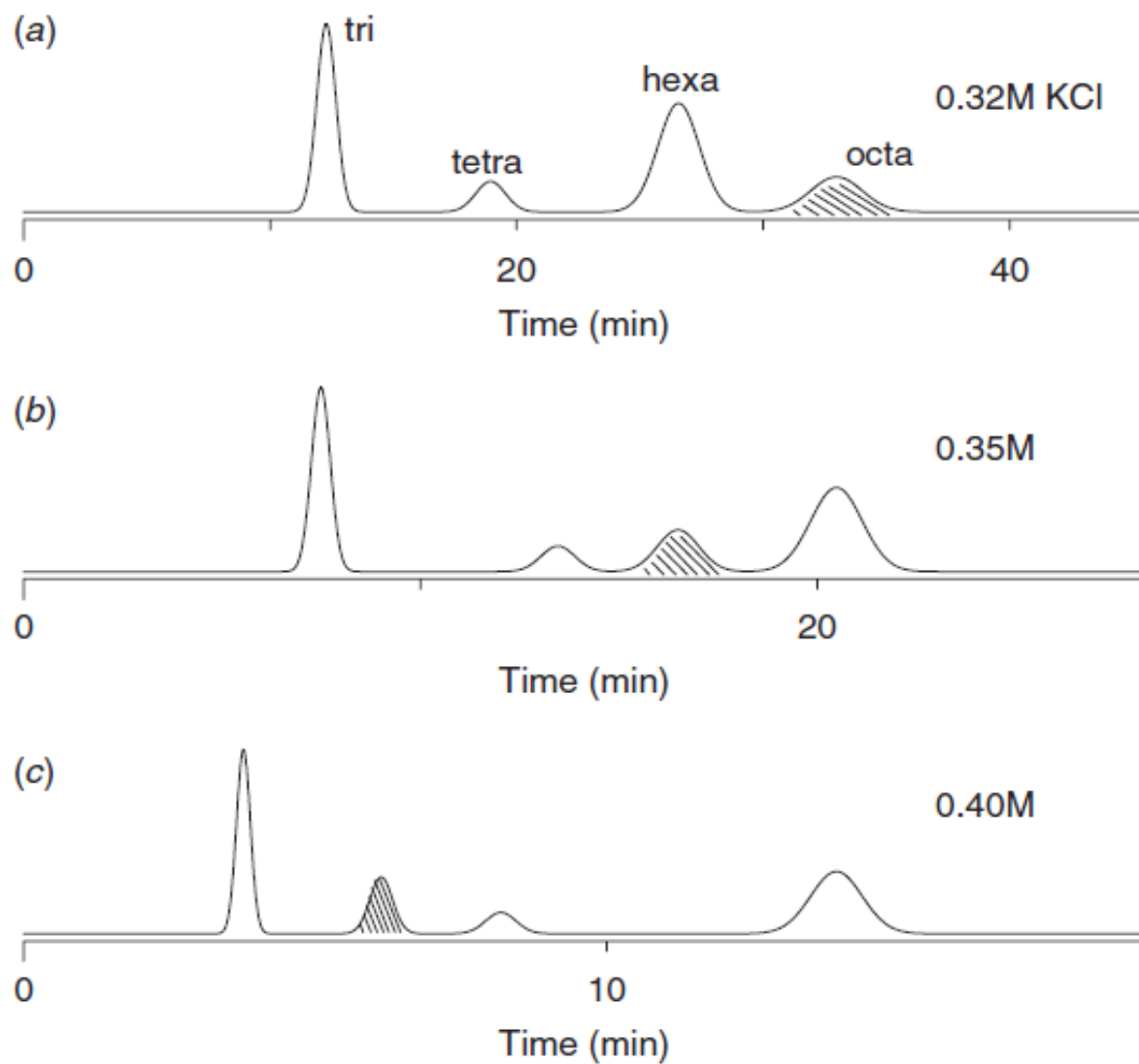
Figure 7.20 Examples of a change in counter-ion (KCl) concentration for the separations of Figure 7.19. Recreated separations for data of [64], assuming a 150 × 4.6-mm column (5-μm particles), 2.0 mL/min, and $N = 1000$.

Mobile-Phase pH

IEC is typically used for acidic or basic samples. As only the charged (ionized) molecule is retained by ion exchange, values of k for a monovalent solute ($m = 1$) will be proportional to the ionization of the solute; for example, as mobile-phase pH is decreased so that an acid goes from fully ionized to half ionized, the value of k will decrease by half. Similarly the ionization and retention of bases will be decreased as mobile-phase pH increases. This behavior is the opposite of retention changes with pH in RPC but is the same as in IPC

IEC Columns

Based on the kind of ionic group R± that forms part of the stationary phase, four general kinds of IEC columns are available: strong and weak anion exchangers (SAX, WAX), and strong and weak cation exchangers (SCX, WCX). Strong IEC columns contain groups R± that are completely ionized over the usual pH range of interest ($2 \leq pH \leq 13$). For strong anion-exchange columns, the most commonly used group R+ is $-N(CH3) +3$; for strong cation-exchange columns, the most common group R− is $-SO −3$ .Weak IEC columns contain groups R± with pKa values in an intermediate range (e.g., $4 \leq pKa \leq 10$); consequently the ionization of these groups (and the ionexchange capacity of the column) can change with mobile-phase pH

Types of IEC….

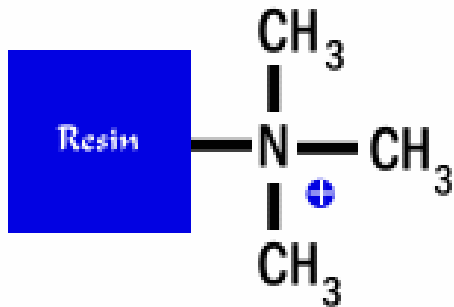➢anion exchangers

➢cation exchangers

Cation exchange chromatography

---positively charged molecules are attracted to a negatively charged solid support. Commonly used cation exchange resins are S-resin, sulfate derivatives; and CM resins, carboxylate derived ions

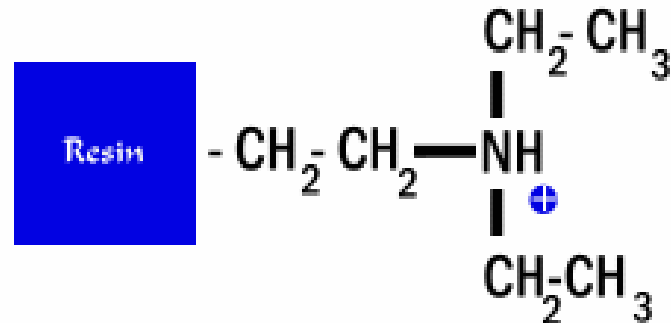S-cation exchanger   CM-cation exchanger
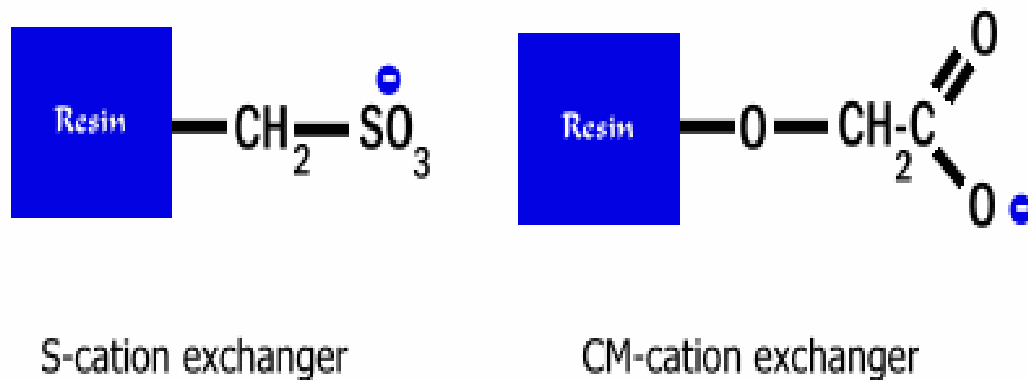
Anion exchange chromatography

---negatively charged molecules is attracted to a positively charged solid support. Commonly used anion exchange resins are Q-resin, a Quaternary amine; and DEAE resin, DiEthylAminoEthane

Q-anion exchanger

DEAE-anion exchanger

Buffers Used In IEC

✓Buffer system 1 : Buffer A = 20 mM Tris, pH=8. Buffer B = 20 mM Tris, 1 M NaCl, pH=8.0

✓Buffer system 2: (Common CEC buffer system): Buffer A = 30 mM sodium acetate, pH=4.5. Buffer B = 30 mM sodium acetate, 1 M NaCl, pH=4

✓Buffer system 3: (AEC for proteins which are very insoluble or have a very high pI)

Buffer A = 30 mM Ethanolamine, 8M urea, pH=10.0

Buffer B = 30 mM Ethanolamine, 8M urea, 1 M NaCl, pH=10.0
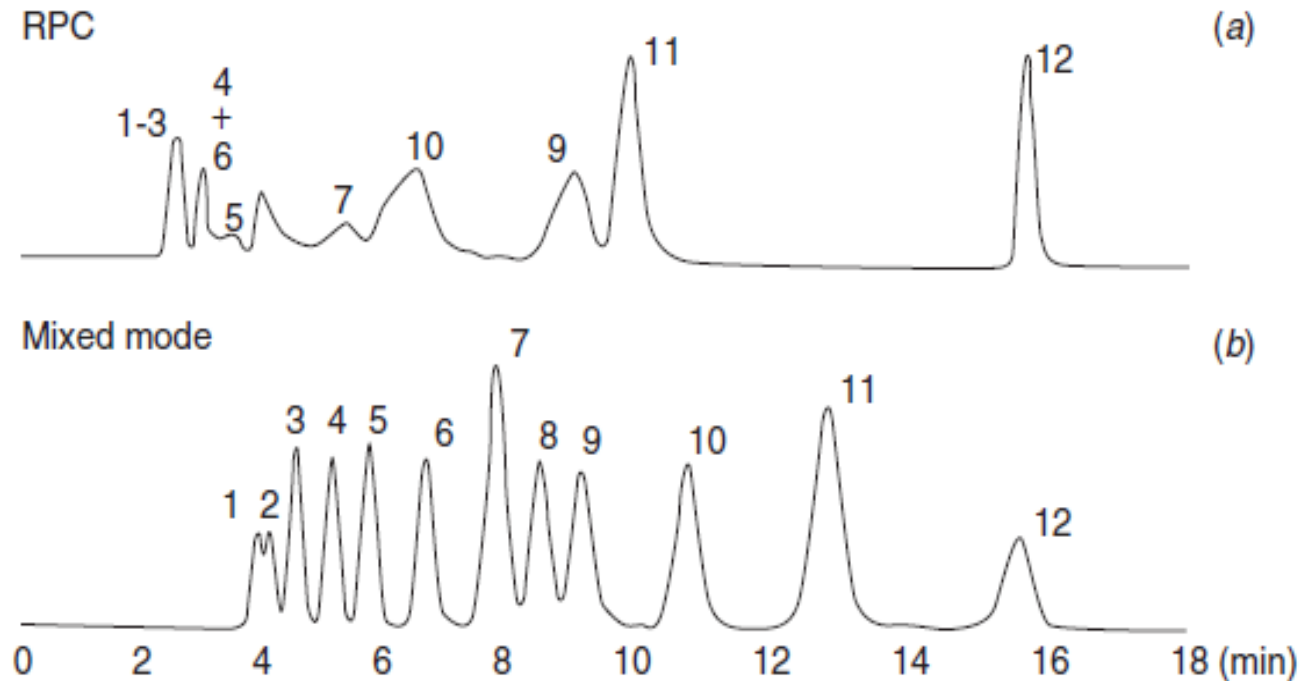
Mixed-Mode Separations

Figure 7.22 Separation of a nitrogen-mustard mixture by RPC (*a*) versus mixed-mode IPC (*b*). Sample: a mixture of small, hydrophilic amines. Conditions in (*b*): 150 × 2.1-mm Prime-sep 100 column (5-μm particles) (SIELC Technologies, USA); 40% acetonitrile/buffer (0.1% TFA); 0.2 mL/min. Adapted from [70].
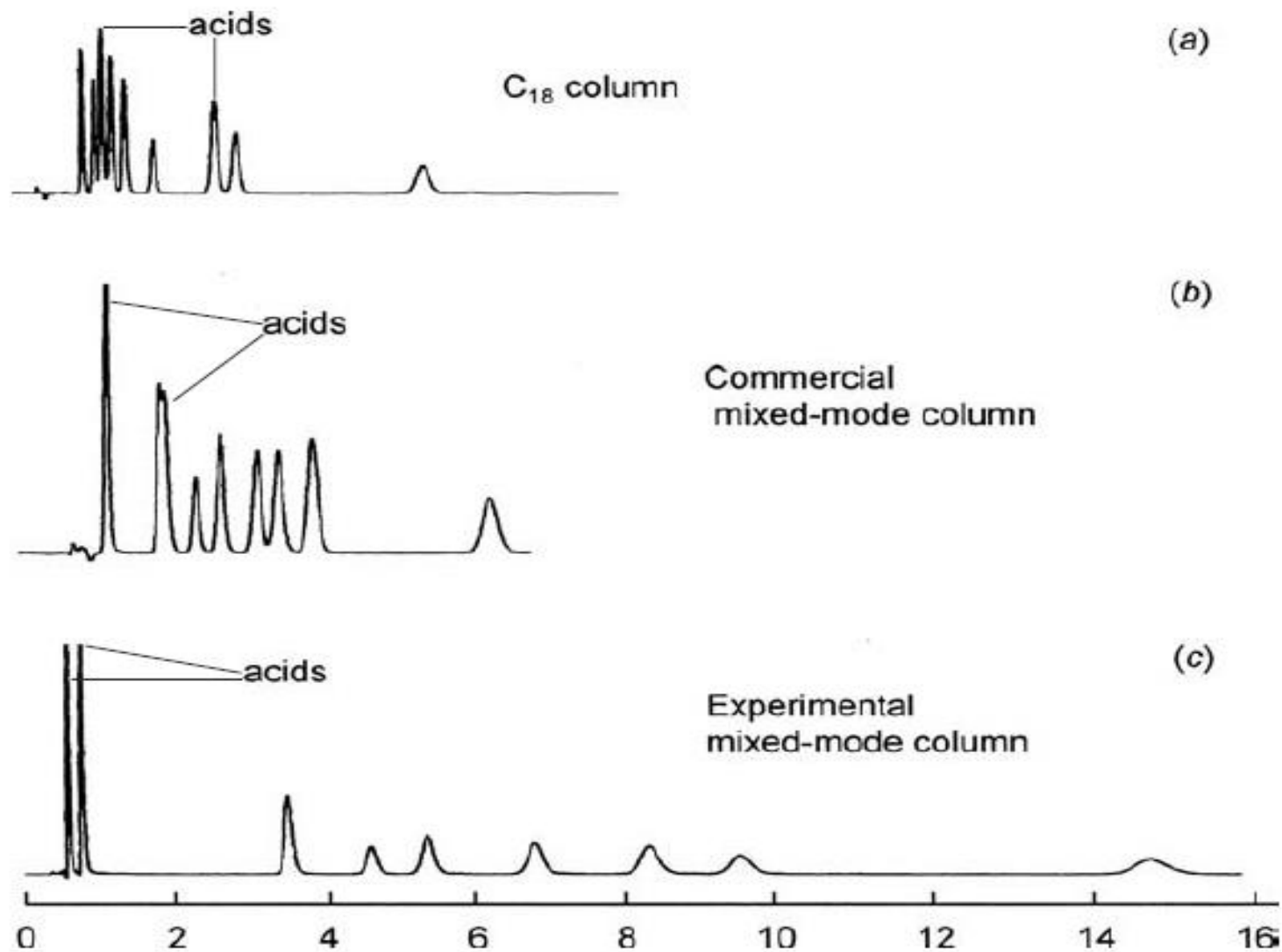
Figure 7.23 Separations of a mixture of acids, bases, and three amino acids by means of different columns. Conditions: 50 × 4.6-mm columns (5-μm particles), 1.0 mL/min; (*a*) C$_{18}$ column, 10% ACN/aqueous 0.1% TFA, 40°C; (*b*) commercial mixed-mode column (PrimeSep 200), 10% ACN/aqueous 0.01% TFA, 40°C; (*c*) experimental mixed-mode column, 24% ACN/0.02% TFA, 65°C. Adapted from [75].

Separations of Carbohydrates

Carbohydrate mixtures can be separated by either hydrophilic interaction chromatography (HILIC) or by IEC. Carbohydrates have pKa values of about 12, which means that high-pH mobile phases can effect their ionization and allow their eparation by anion-exchange chromatography (AEC). AEC separations of carbohydrates is now generally preferred because of the greater sensitivity of amperometric detection (detection limits<1 nanomole), combined with the possibility of influencing selectivity by small changes in mobile-phase pH.
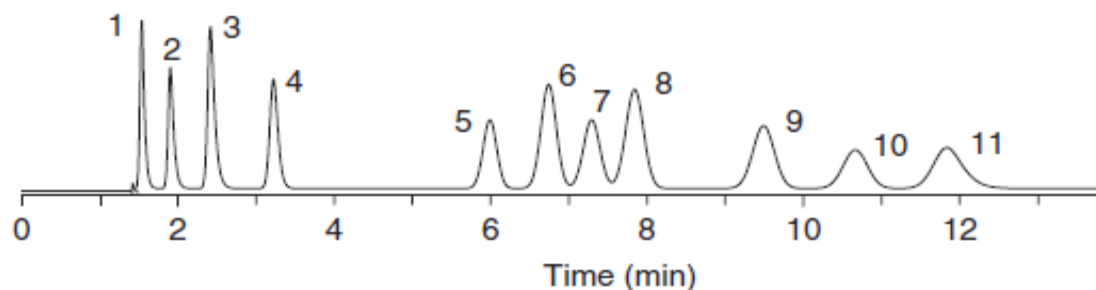

Figure 7.21 Separation of a mixture of carbohydrate standards by anion-exchange chromatography with amperometric detection. Sample: *1, myo*-inositol; *2*, D-sorbitol; *3*, lactitol; *4*, L-fructose; *5*, rhamnose; *6*, D-galactose; *7*, D-glucosamine; *8*, D-glugose; *9*, D-mannose; *10*, D-fructose; *11*, D-ribose. Conditions: 300×4-mm anion-exchange column (5-μm particles); mobile phase, aqueous 5-mM NaOH + 1-mM Ba(OAC)$_2$; ambient temperature; 1 mL/min. Adapted from [68].

Separation of proteins and peptides

- The most important advantages of IEC for protein isolation include (1) the tendency of proteins to maintain their native conformation and biological activity during separation, (2) the relatively high binding capacity of IEC packings, and (3) high mass recoveries.

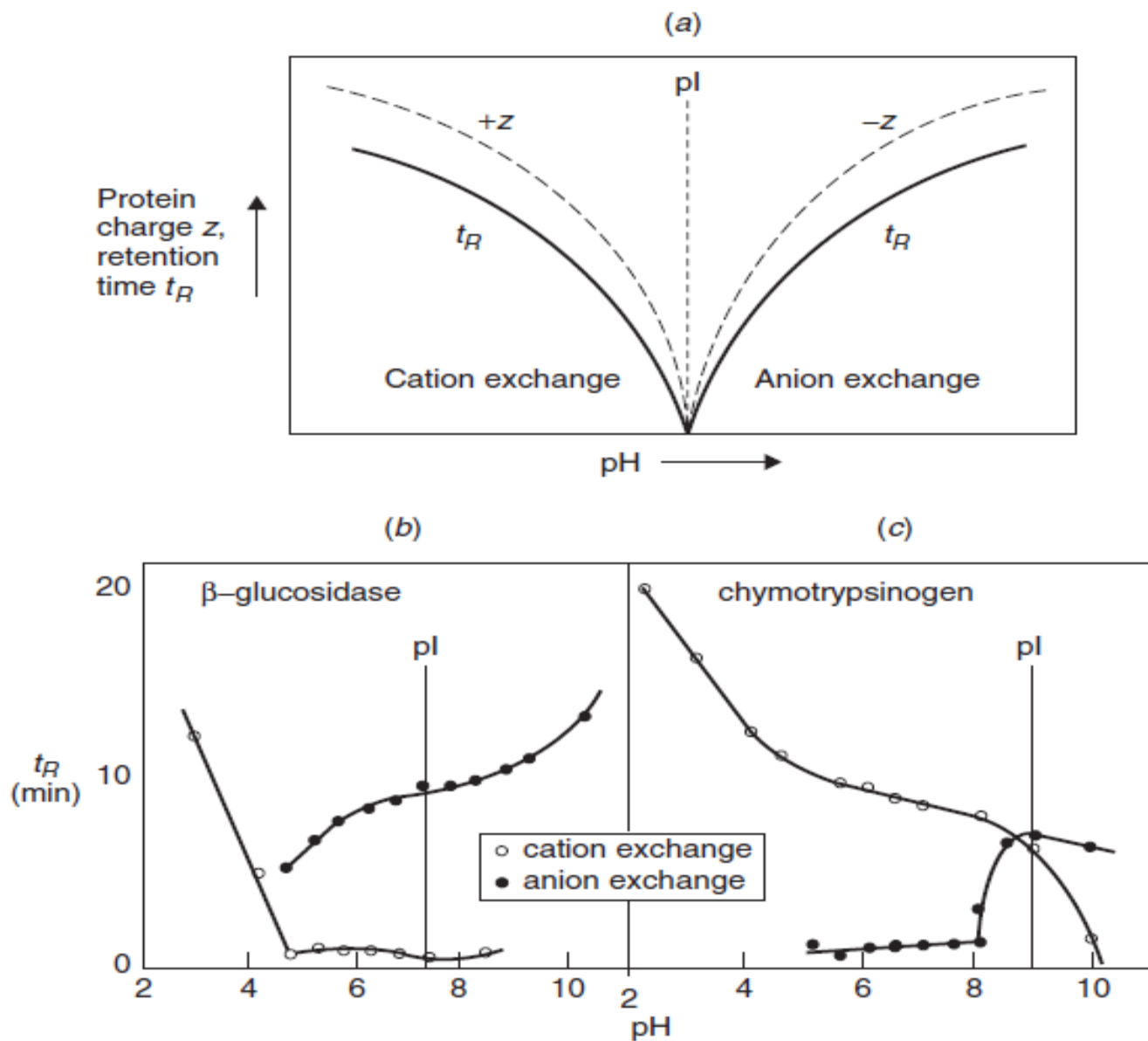| pK$_a$ Values for Charged Amino Acids		
Residue	pK$_a$ in Amino Acid	pK$_a$ in Protein
Terminal amino	8.8–10.8	6.8–7.9
Arginyl	12.5	$\geq$12
Histidyl	6.0	6.4–7.4
Lysyl	10.8	5.9–10.4
Terminal carboxyl	1.8–2.6	3.5–4.3
Aspartyl	3.9	4.0–7.3
Glutamyl	4.3	4.0–7.3

Figure 13.15 Protein retention on ion exchangers as a function of pH. Ideal behavior (*a*); actual behavior of β-glucosidase (*b*) and chymotrypsinogen (*c*). Adapted from [38].
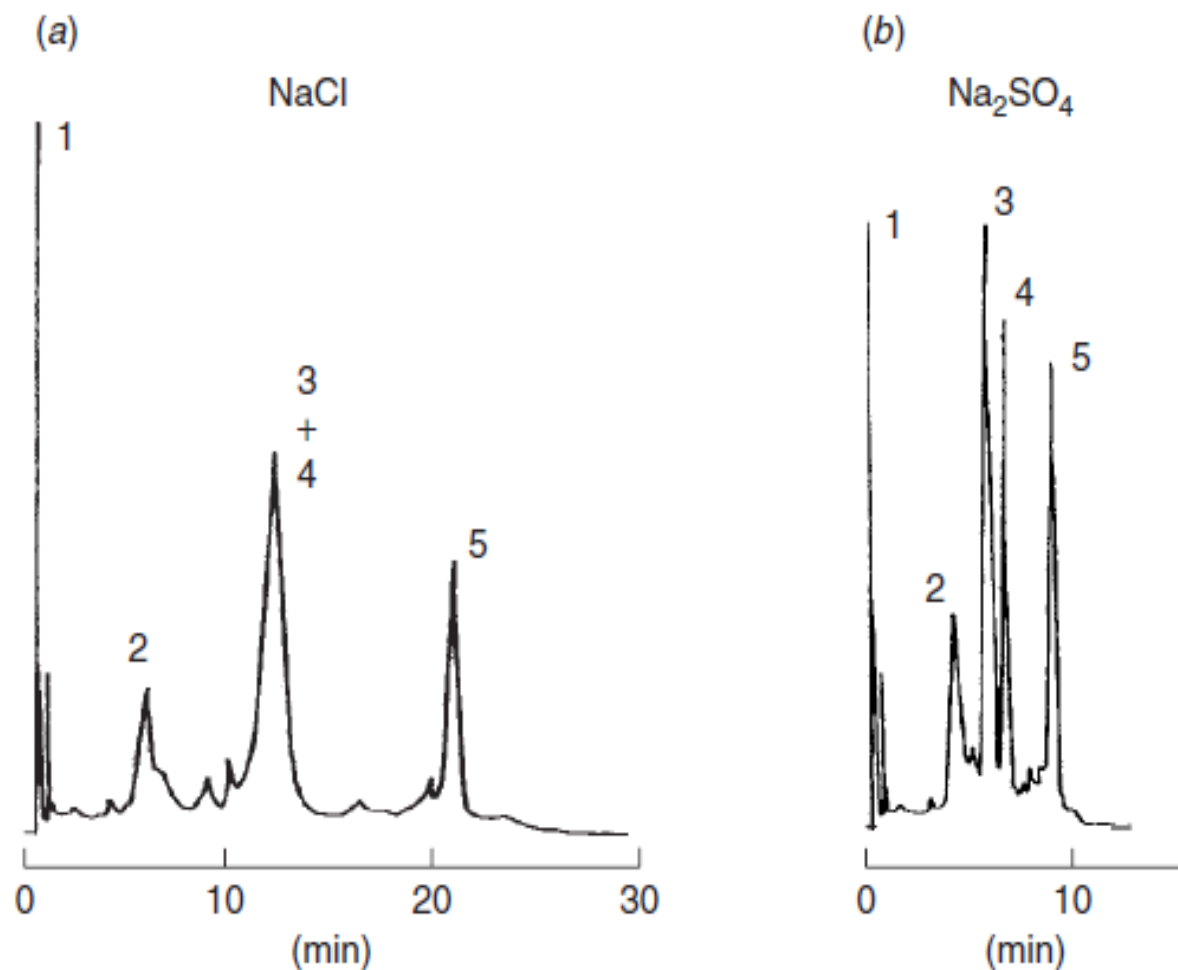
Figure 13.17 Effect of salt type on anion exchange separation of five proteins. Conditions: 50 × 4-mm Shim-pack WAX-2 column (Shimadzu); 0−0.5M of indicated salt in 20 min; pH-8 phosphate buffer; 1 mL/min. Adapted from [41].

Hydroxyapatite Chromatography
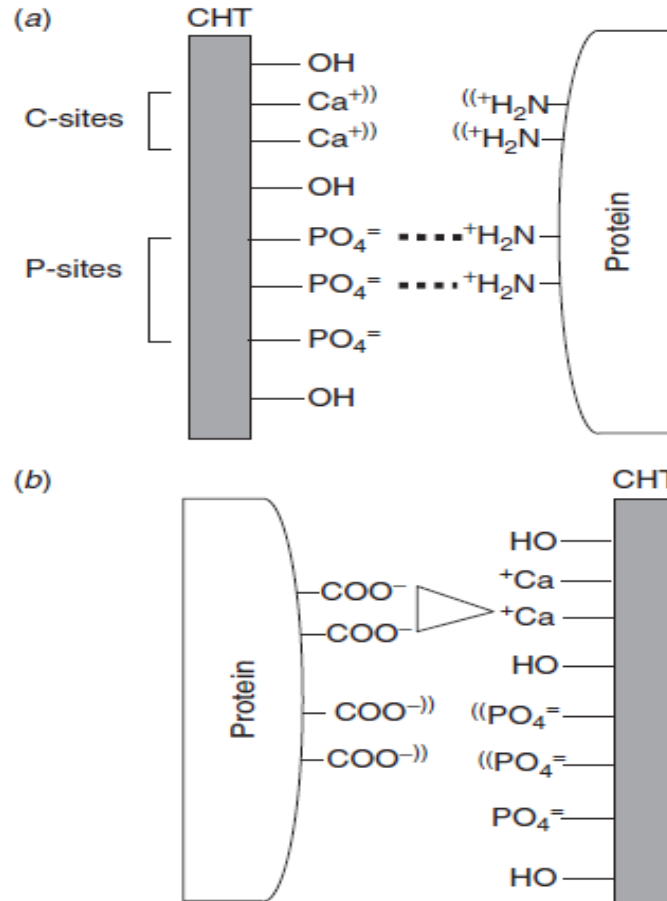
Figure 13.18 Binding to ceramic hydroxyapatite (CHT) of a basic protein (*a*) and an acidic protein (*b*). Double parenthesis indicate repulsion, dotted lines indicate ionic bonds, and triangular linkages indicate coordination bonds. Adapted from [37].

Downstream processing and chromatography based analytical methods for production of vaccines, gene therapy vectors, and bacteriophages

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Since chromatography based tools for purification of biopharmaceuticals were optimized for purification of smaller molecules and proteins, purification of virus particles was carried out mainly by using other techniques (density gradient ultracentrifugation, precipitation, etc.).

Poor scalability of these processes, sometimes inadequate purity of the product and/or poor economics together with the recognized potential of gene therapy and higher regulatory requirements regarding the purity of virus preparations used for vaccination led to the development of new and advanced technologies in the field of chromatography media.

Ultracentrifugation of viruses can be carried out in different density gradients like caesium chloride,[21] sucrose[22] or iodixanol.[23,24] In general the method enables efficient purification and concentration of virus particles in one step, but requires expensive equipment, process times are long (with subsequent step of density gradient material removal by dialysis or size exclusion chromatography) and sometimes several cycles are needed[21,25] to reach the required virus purity. In addition, some density gradients are not suitable for certain viruses. For example, high viscosity and hyper-osmotic property of sucrose can cause damage to the extremely labile virus[24] leading to loss of virus infectivity and unsatisfactory infectious virus yields.[25] Another problem is extensive virus aggregation during gradient ultracentrifugation which was observed by Peng et al.[23] when trying to purify recombinant adeno vectors in CsCl density gradient.

Selective precipitation uses different chemical agents like ammonium sulfate and polyethylene glycol25 to precipitate either the virus or the impurities. However, this method is not very suitable for preparative scale virus purification/production; it is especially challenging to carry out this method in a batch mode under cGMP conditions. Both, density gradient ultracentrifugation and selective precipitation are difficult to scale up and can suffer from low virus yields.

Ultrafiltration, a filtration method based on molecular weight differences between the target compound and impurities can be designed as a very robust method resulting in high virus yields. The scale up of the method to the industrial level is relatively straightforward and if coupled with diafiltration also provides a buffer exchange step. However, precipitation and ultrafiltration alone cannot deliver a product of sufficient purity and need to be combined with other techniques.

Dynamic binding capacity and virus recovery on different chromatography supports for different virus (-like) materials.

Nanoparticle	Type	Capacity	Recovery	Reference
Influenza	CIM QA monolith	2E + 10 TCID$_{50}$/mL	70%–87%	[11]
			42%	[12]
	Mustang Q	2E + 10 TCID$_{50}$/mL	40%	[12]
	Q Sepharose XL	1E + 9 TCID$_{50}$/mL	24%	[12]
Adenovirus 5	Q Sepharose XL	4E + 13 VP/mL	n/d	[4]
	Sartobind Q	1.5E + 13 VP/mL	60%	[73]
Canine Ad 2 (CAV2)	Fractogel EMD propyl (capture)	4.5E + 10 VGCN/mL	77%–100%	[8]
	CIM DEAE monolith (polishing)	7E + 11 viral VGCN/mL	58%–77%	[8]
Adenovirus 3 VLP	CIM QA monolith	1.3E + 16 VP/mL	52%	[42]
Lentivirus	CIM DEAE monolith	>8E + 7 IP/mL	80%	[10]
	Q membrane	n/d	70%	[74]
Retroviral vectors	DEAE Sepharose FF	>3E + 7 cfu/mL	53%	[75]
Flavivirus	CIM QA monolith	4.5E + 9 FFU/mL	72%	[76]
	Mustang Q membrane	n/d	36%	[76]
	Capto Q resin	n/d	37%	[76]
Baculovirus	CIM QA monolith (capture)	2.4E + 11 pfu/ml	up to 99%	[77]
	Sartobind D membrane	7.7E + 10 TP/mL	65%	[78]
	Capto DEAE resin	n/d	33%	[78]
Rotavirus	CIM QA monolith	4E + 12 VP/mL	n/d	[79]
Rotavirus like particle	Resource Q	n/d	50%	[80]
Rotavirus like particle	Sartobind D membrane (capture)	n/d	55%	[81]
Rotavirus like particle	Sartobind D membrane (concentration)	n/d	97%	[81]
T7 bacteriophage	CIM QA monolith	1E + 13 pfu/mL	100%	[25]
M13 bacteriophage	CIM QA monolith	4.5E + 13 pfu/mL	100%	[25]
Lambda bacteriophage	CIM QA monolith	1E + 13 pfu/mL	100%	[25]
PRD1 bacteriophage	CIM DEAE/QA monolith	6E + 13 pfu/ml	60%–80%	[27]
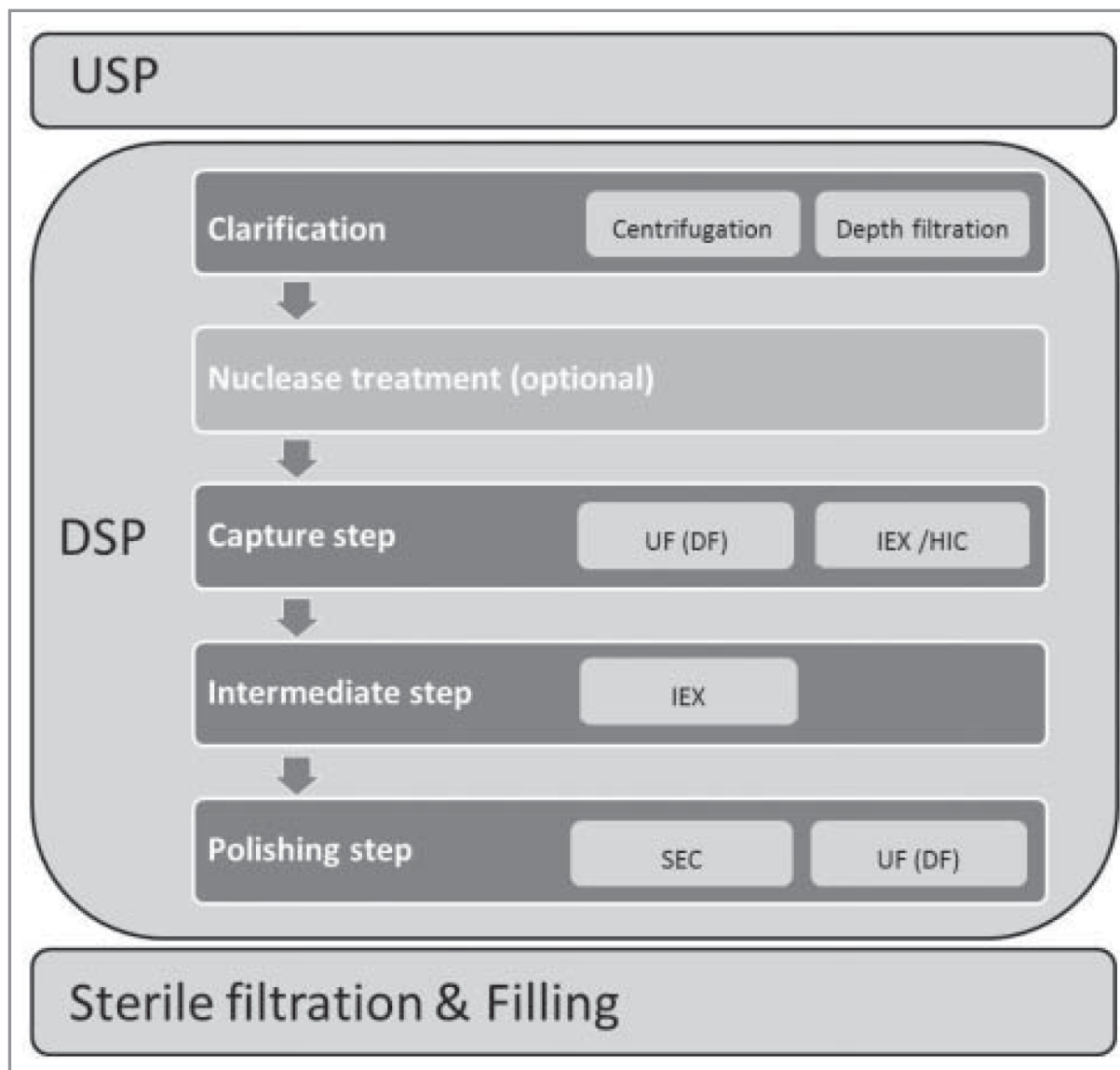
General scheme of virus production process with emphasis on DSP part. Abbreviations: UF-ultrafiltration; DF-diafiltration; IEX-ion exchange; HIC-hydrophobic interaction mode; SEC-size exclusion chromatography.

Current recommendations regarding the level of residual cell-substrate DNA

for vaccines produced in cell lines derived from human tumors are ≤10 ng per dose and a

median DNA size of 200 bp or lower.

The most widely used chromatography mode for the capture step of industrial scale virus DSP is charge specific separation where viruses are separated from impurities on the basis of the difference in their surface charge distribution. Since majority of viruses have isoelectric points below 6, they efficiently bind to anion exchange matrixes at neutral pH. However, also majority of contaminants bind to anion exchangers at neutral pH, therefore the separation of viruses from impurities is performed by selective elution. Elution is carried out by *changing the pH* of the buffer (not widely used as viruses are pH sensitive) or by increasing the *ionic strength* of the buffer (salt concentration in the buffer).

Viruses bind to cation exchangers **when** low pH buffers (below their isoelectric point) are used, but for human viruses, as they are sensitive to low pH, this usually is not an option. However, certain phages are more stable in low pH environment 34 and in these cases using a cation exchanger is of great advantage, because host cell DNA (a very important impurity needed to be removed) does not bind to the cation exchanger and is therefore removed very efficiently.

However, if the chromatography step enables efficient HC DNA removal it is preferred that no deoxiribonuclease is used in the DSP as DNA fragmentation and size reduction can increase DNA content in the final virus pool after chromatography step. Additionally, deoxiribonuclease has to be removed from the final product intended for human use. The incorporation of DNA degradation step into the downstream process of phages was studied by Smrekar et al.44 They have investigated the influence of DNase on the purification process of lytic phages and reported that addition of DNase lowers process productivity because of long degradation time and due to the risk of non-completed DNA degradation resulting in co-elution of the phage and DNA fragments.

The last step in the DSP (polishing step, SEC) should provide final impurity removal, additional concentration of the active substance (nanoparticle) and buffer exchange into final formulation. This final step can be performed by applying SEC _or_ UF/DF. SEC is usually performed in a group mode separation where the virus elutes in the void volume, while all remaining low molecular weight impurities elute later on. Optimized SEC method can result in high virus yields and it is very efficient in simultaneous removal of residual impurities and buffer exchange.13 Bandeira et al.10 increased lentivirus yield with optimization of SEC as a polishing step from 27% to 70% and up to 100% virus yields were reported for Influenza virus purification with SEC.13,37 However, SEC does not enable virus concentration and there are limitations regarding the flow rates of the mobile phases and the loading volume of the sample; usually up to a few percentages of the SEC column volume can be loaded in a single run.

On the other hand UF/DF does not have these limitations and also enables final concentration of the virus and it is therefore used in many virus DSPs.

At the end of the virus DSP additional sterile filtration step can be introduced; UF concentrate or SEC eluate is filtered through 0.2 µm filter for final removal of bioburden.

Analytical Monitoring
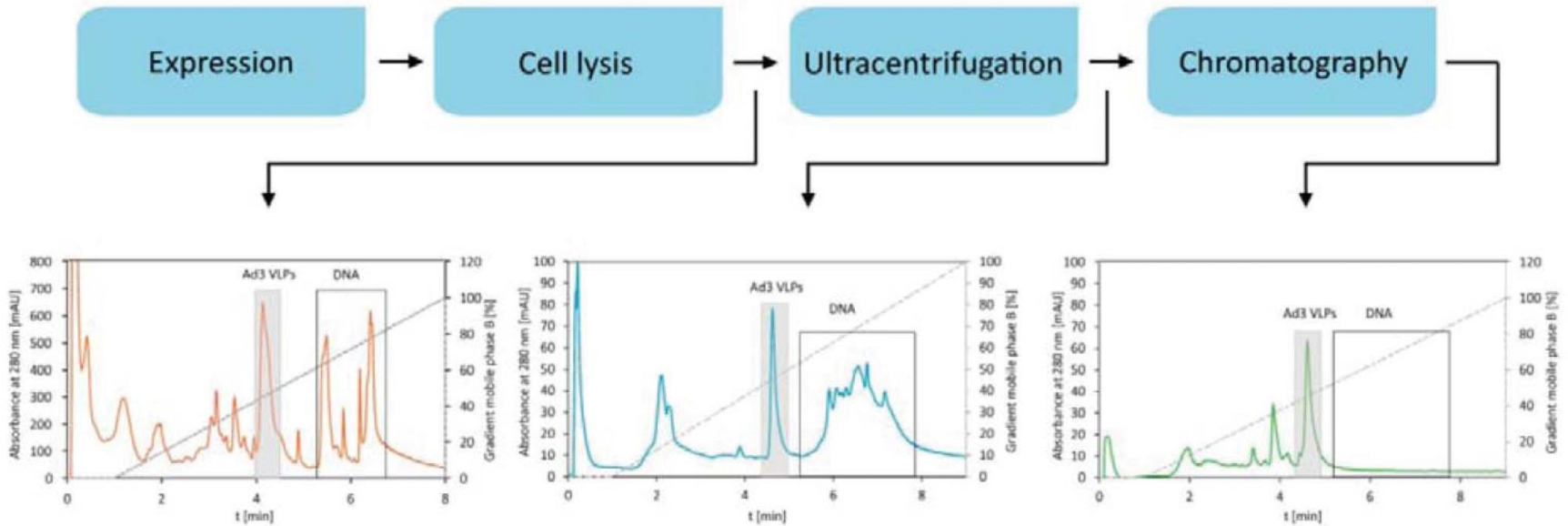
Fingerprint HPLC elution profiles of Ad3 VLPs purification process. Elution profiles depict the purity/impurity profiles of samples containing Ad3 VLPs after particular DSP steps.

Review

Recent advances in chromatographic purification of plasmid DNA for gene therapy and DNA vaccines: A review

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DNA vaccination is the use of DNA particles of pathogens to induce the immunity against certain contagions. It is a novel sort of vaccination methods [11,12], which was identified as cellular and humoral immune responses stimulant. The new vaccination method became available for clinical trials after just ten years of its debut [13], and some of DNA vaccines are now available in the market like West Nile virus vaccine which is used for veterinary applications [14].

On the other hand, plasmid DNA (pDNA) vaccines are a subtype of DNA vaccines, where the plasmid encoding certain protein antigen was extracted from the pathogen cell and transformed into *Escherichia coli (E.coli)* [15]. The plasmids replicate in the *E.coli* cells in a process called "Up-Stream process". After that, a process of downstream is applied to obtain a purified amount of plasmids [16]. The purified plasmids are injected into the human body and reach the nucleus of human cells by endosomal trafficking.

Immobilized-Metal Affinity Chromatography (IMAC)

Initial column

matrix

chelator

ligand

**Step 1.
load metal**

$+$ Cu^{++} $\longrightarrow$ Cu^{++}

$7 < pH < 8$
$+$
P

**Step 2.
Bind protein (P)**

$Cu^{++} + P$ $\xleftarrow{\;4 < pH < 5\;}$ $Cu^{++} \cdots P$
**Step 3
Desorb protein**

$+ EDTA$ | **Step 4
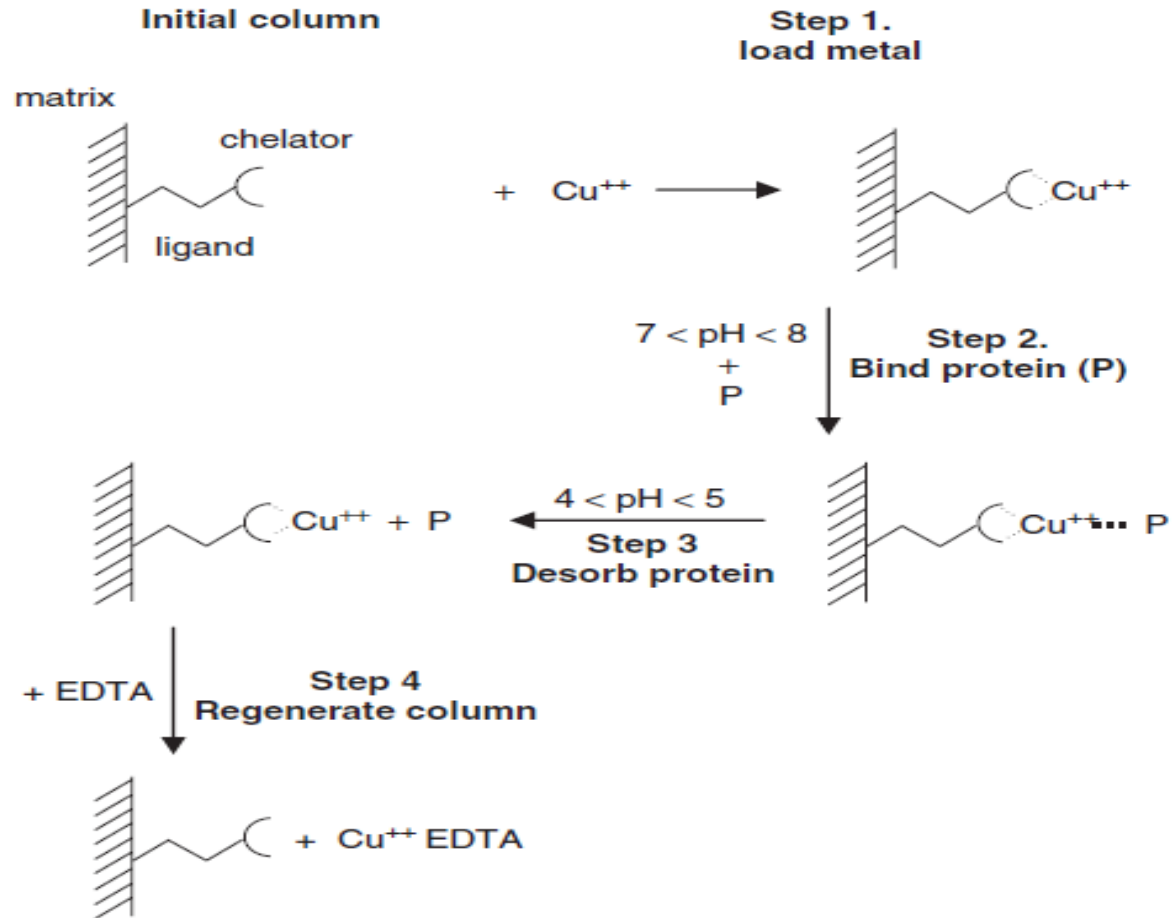Regenerate column**

$+ Cu^{++} EDTA$

Figure 13.19 Steps in the use of IMAC. Adapted from [56].

- The strength of metal binding by different amino-acid groups in the protein moleculedecreases in the following order:

$$his>trp>tyr>phe>arg \sim met \sim gly$$

Selectivity in IMAC can be controlled by the choice of:

- chelating ligand

- immobilized metal ion

- mobile-phase pH and ionic strength

- any mobile-phase additives used to enhance binding or elute proteins