



A REVIEW ON HPLC COLUMN CHARACTERISTICS AND SPECIFICATION

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1. ABSTRACT

The column is an two types reversed phase and normal phase column. The column packed with different particle size of stationary phases. It often an efficiency of column. E.g. 5 μ m porous particle size is best suited for number of solutes. The small particle size(3 μ m, 5 μ m), longer length(25cm) of column shows more number of theoretical plates, plate number more than 2000 ie, more efficient column. For column packing mostly silica use because its high mechanical strength. In bonded phase the sterically protected organic groups attached to silane group gives less concentration of silane group shows more efficiency of bonded phase. Peak asymmetry depend on column particle size, equal sphericle size particle gives asymmetry value less than 1.5.

2. KEYWORDS: Column, HPLC, triethylamine, porous particle, theoretical plate number, bonded phase silica.

3. INTRODUCTION

The column is the heart of HPLC separation processes. The availability of a stable, high performance of the column is essential in developing a rugged, reproducible method. Commercial columns can differ widely among suppliers. Such differences can serious impact on developing the desired HPLC method. Specifically, different columns can vary in plate number, band symmetry, band spacing and lifetime. The column is an cylindrical long tube. Made from stainless steel, glass etc. and packed with either solid or liquid mobile phases.the column packed with solid material known as solid liquid chromatography and packed with liquid stationary phase known as liquid-liquid chromatography.

The columns are two types reversed phase HPLC columns and Normal phase HPLC columns. In reversed phase column the stationary phase is nonpolar and mobile phase polar, in normal phase column the stationary phase is polar and mobile phase is nonpolar.

4. Characteristics of column and column packing

4.1. column packing particles

Most column packings used for HPLC separations. Different types of column packing particles are following type

- Micropellicular particles
- Totally porous microspheres
- Perfusion particle

1) Micropellicular particles: have a solid core with a very thin outer skin of interactive stationary phase.

These silica or polymer based particles, usually available in 1.5 to 2.5 μ m sizes, display outstanding efficiency of macromolecules because of fast mass transfer kinetics. These solid ultramicroparticles have limited sample load characteristics because of low surface areas, thus best suited for analysis only.

- 2) Totally porous microspheres: these are most commonly used because of the favorable compromise pH desired properties: efficiency, sample loading, durability, convenience and availability. These particles are available in a variety of diameters, pore sizes, and surface areas, so that all types of HPLC methods can be developed with this material.
- 3) Perfusion particles: It contains very large pores (e.g. 4000 to 8000Å) throughout the support and also include a network of small interconnecting pores (e.g. 300 to 1000 Å) between these large through pores. At a high flow rates, solutes can enter this pore structure through a combination of convective and diffusion. This effect minimizes band broadening, so that large porous particles resemble smaller particles in terms of column efficiency.

Particle size is very important in HPLC. Particle diameters of about 5 μ m represent a good compromise for analytical columns in terms of column efficiency, backpressure and lifetime.

4.1.1 Silica packing particles

Silica based packings presently the most popular HPLC column packing materials. Totally porous silica particles

can be prepared with a narrow pore size distribution in a wide choice of pore sizes (8, 30, 100 nm) and particle sizes (e.g. 10,5,3 μ m). A strong advantage of most silica particles is their high mechanical strength. This permits the formation of efficient packing beds that are stable under high operating pressures for long periods. Although chromatographic silica is available in both spherical and irregular shaped particles. The spherical particles have more advantages over irregular particles, i.e. more efficient, easily packed etc.

A desirable property of silica support is that the surface can be chemically modified with a large variety of bonded phase having different functionalities. This feature permits the formation of packed beds that are stable during use with various solvent types and during gradient elution.

However, silica is not a perfect support for hplc columns. An unfavorable characteristic of silica is its solubility at high pH. For satisfactory lifetime sil-gel technique use, in which the precipitation of The hydrated silica surface can contain various kinds of silanol groups, as illustrated in fig. 1.1. However, these free silanols can cause strong, deleterious binding of basic solutes because of their acidic nature. Therefore, silicas with a higher concentration of free, more acidic silanols often show increased retention and broad, tailing peaks for basic samples.

Geminal silanols are less acidic than isolated silanols and generally are friendly for separating of basic solutes.

The purity of silica support is main concern because when silica contaminated with certain metals (Fe, Al, Zr, Ni etc) can form complex chelating solutes. Causing asymmetrical or tailing peaks, or completely retaining compounds so that elution does not occur.

Columns packed with porous, polymeric particles can also be useful for developing HPLC methods. Some of these polymer particles (polystyrene) are hydrophobic, meaning that they can be used directly for reversed-phase separations without addition of a surface coating agent. Another ex. Divinylbenzene-cross-linked polystyrene, methacrylates, polyvinyl etc.

The main advantage of porous polymers is that they are applicable in the pH range 1 to 13. i.e. these columns are useful in separating highly basic solutes. These column packings materials are most used for separating and isolating materials from biological sources. In such applications, porous polymers have an advantages of longer stability, compared to silica based columns.

Porous Polymers: Columns packed with porous, polymeric particles can also be useful for developing HPLC methods. Most polymer particles for reversed-phase HPLC are made of divinylbenzene-cross-linked polystyrene polymer, polyvinyl alcohols etc.

The main advantages of porous polymers is that they are applicable in the pH range 1 to 13.

Limitations: Lower column efficiency than silica-based columns with same particle size.

A special problem with polymer columns is that this support swells differently in the presence of various organic modifiers.

4.1.2 Other inorganic support

1) These columns generally are useful for specific applications because of special properties.

Graphitized Carbon: Underivatized, graphitized carbon is prepared synthetically in porous spheres with various particle sizes. The surface of the graphitized carbon provides the basis for retention-no other stationary phase is required.

Graphitized carbon has proved useful for separating certain geometric isomers. These columns are also useful for retaining and separating compounds that are too highly hydrophilic for adequate retention on C18 bonded phase column.

Advantage: These columns are useful at any temperature and pH.

Limitation: The column has lower efficiency and higher fragility compared to silica particles.

Obtaining good peak shapes can also be a problem with some mobile/solute systems, particularly compounds with longer K values.

The columns are available only in short length and are expensive, a variety of pore sizes are not available at this time.

Alumina: Both narrow and wide range pore alumina particles are available for HPLC. Alumina is produced in different particle sizes but not such as chromatographic silica.

Advantage

Alumina based packings useful with mobile phase pH-12.

Highly basic compounds can be separated using high pH mobile phase without ion-pairing agents.

Limitation: Consequently alumina columns have not reached the general level of acceptance of popular silica based units.

Zirconia: Columns of polymer coated porous zirconia microspheres are commercially available now.

The strong packings are useful with all known HPLC eluants throughout the pH range 1 to 14 and at temperature 100°C.

4.2 Column Configuration

In HPLC different types of column used ex. Glass, stainless steel (ss) etc. Most commonly for hplc method development use straight lengths of stainless steel (ss) tubing with highly polished interior walls. SS is useful with all organic solvent and most aqueous buffers. However, chloride containing mobile phases can slowly cause halide cracking of the SS (at low pH).

Commercial columns made from glass, glass-lined SS and plastic are available for sample might interact deleteriously with SS.

The surface area of the column internal wall is quiet small, so the opportunity for interaction is less.

Column configurations (stainless steel)

Type	Inner diameter (cm)	Length(cm)	Particle size(µm)
Analytical			
Compression fittings	0.3-0.46	3-25	3-10
Cartridge	0.3-0.46	7.5,10	3-10
Microbore	0.1-0.21	15,25	3-8
Semipreparative	0.8-1.0	10-25	5-20
Preparative	2.0-5.0	10-25	5-20

Table summarizes column configurations that are commercially available for column packings. Analytical methods usually are best developed with 0.46 to 0.3 cm ID columns having particles in the range 3 to 10 µm. columns of 5-µm particles generally give the best compromise of efficiency, reproducibility and reliability.

Columns of 3-µm particles allow faster separations or higher efficiency but have tendency plug more easily, which reduces column lifetime.

4.3 Stationary Phases

4.3.1 Bonded Silanes: Silica-based reversed phase packings typically are made by covalently bonding an organosilane or by depositing a polymeric organic layer on the support surface. Most widely used are packings with surface-reacted organosilanes using the reactions in following fig.

Many manufacturers attempt to densely(completely) react the silica surface with silane. However, because of the steric bulk of the bonded phase ligands, all of the silanol groups on the surface can not be reacted. The % of silanol reacted groups decreases even with the smallest silane(trimethyl or C₁), almost 50% Of the silanol groups unreacted on the surface. So use C₁₈ to minimize silanol groups concentration of bonded phase increases.

Glass columns recommended by some for samples of biological origin are pressure limited (ex. Less than 1000 psi) and restricted in their application.

Rigid polymer columns with polymer fittings are available for applications where other materials are not appropriate. These columns have aluminium outer shells that provide additional strengths at high operating pressures.

Columns with soft outer polymer shells are also available. These columns are available for both analytical and preparative applications. the advantage of this column type is lower cost and a nonmetal construction.

Alternatively, less costly SS cartridge columns also are available in various dimensions with a wide range of packing materials.

End capping: So manufacturers of column with silane bonded phases(C₈,C₁₈) use a process called endcapping to fully react(silanize) the silica support surface. This approach increases coverage of the support by reacting residual silanol groups to minimize unwanted interactions with solutes. However, endcapping cannot completely overcome the disadvantages of an acidic silica support. Endcapped columns may be more stable at intermediate and higher pH(6 to 9) because of better protection of silica support against dissolution.

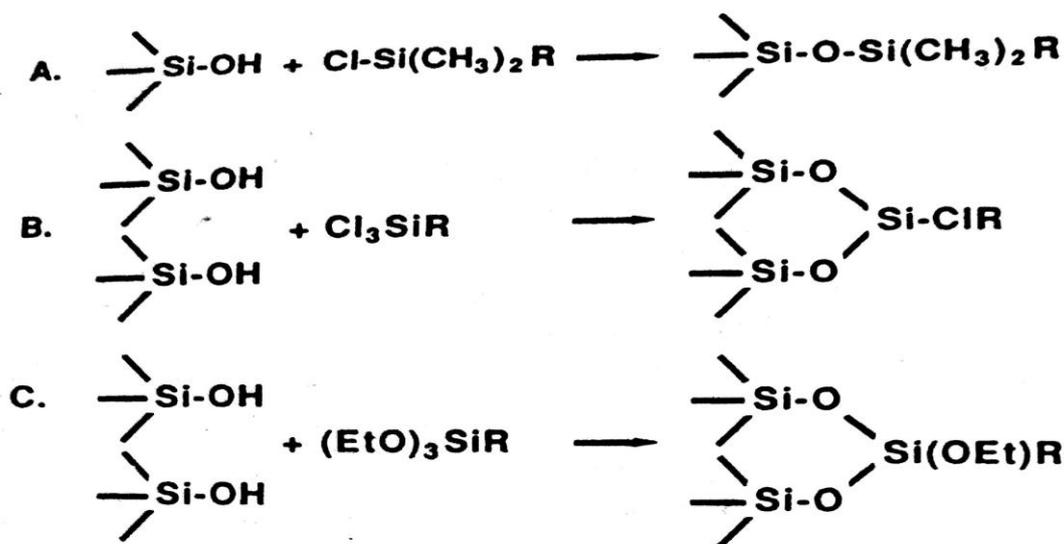


Figure 1: Chemistry of bonded phase packing (a) Reaction of surface silanol with chloromethylsilane; (b) reactions of surface silanols with trifunctional silane; (c) reactions of surface silanols with trifunctional alkoxy silane.

4.3.2 Other Stationary Phases: Other methods of covalently attaching organic stationary phases have been reported, but commercial products based on these procedures are not new available. Some column packings contain stationary phase prepared by polymerizing various monomers on a support. Polybutadiene-modified alumina and zirconia column packings and other polymeric stationary phases have been commercialized.

4.3.3 Retention of the bonded phase in reverse phase column: The surface area of the bonded phase support is a major factor; the larger the surface area, the greater the retention (k). For separations involving only hydrophobic interactions, retention tends to increase with percent carbon.

Sample retention normally increases for bonded phases of greater length ($\text{C}_{18} > \text{C}_8 > \text{C}_3 > \text{C}_1$), but there is not much difference among long-chain packings (i.e. $\text{C}_8 = \text{C}_{18}$).

4.3.4 Stability of Bonded-phase Columns: The stability of bonded-phase packings is especially important in different types of solute separations. Longer-chain alkyl-bonded-phase packings (e.g. C_8 and C_{18}) are more stable than short-chain bonded phases. The stability (and lifetime) of silica-based bonded-phase columns is directly proportional to the types of silica supports and bonded phases. From hydrolysis of the Si-O-Si bond that binds the silane to the support. This degradation is accentuated at higher temperatures, low pH, and highly aqueous mobile phases.

Another way to improve the stability of stationary phases at low pH is to use sterically protected functional groups. Bulky monomeric silanes can minimize the hydrolysis of a silane covalently attach to the silica support. Each Si-O-Si bond is individually protected because of the size

of the two isobutyl groups is known well in solution chemistry.

To minimize silica support dissolution:

- Less-soluble supports made from sol-gel silica's
- Densely bonded, highly end capped packing's with longer-chain alkyl legends
- Organic or borate buffers (avoid phosphate and carbonate)
- Operating temperature of $\leq 40^\circ\text{C}$

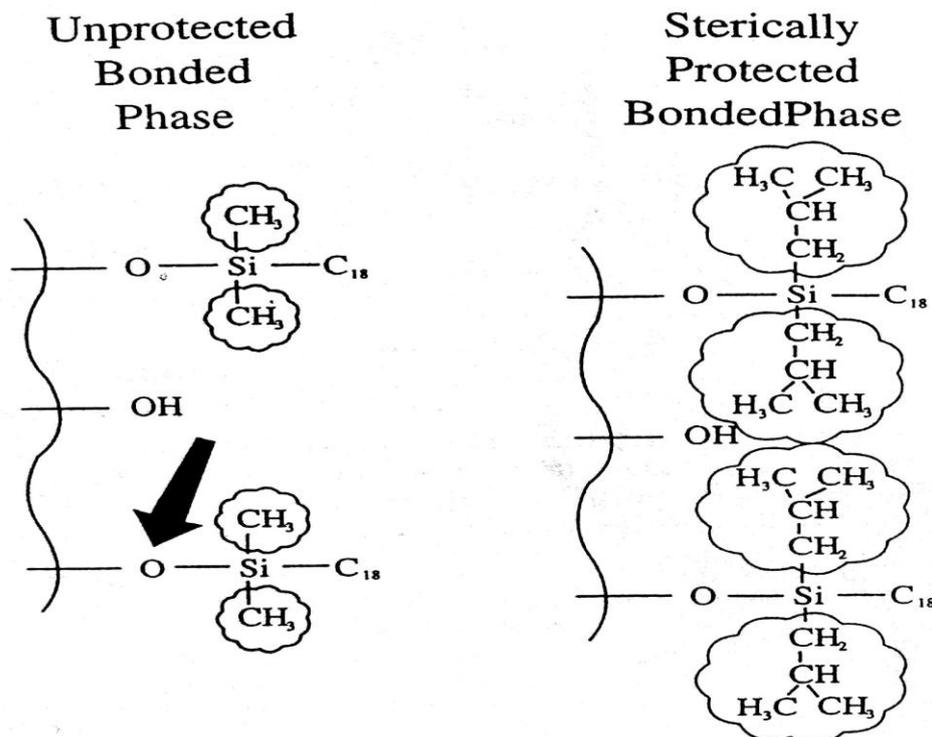


Fig. 2: Hydrolysis of Si – O – Si bond of silane bonded phases.

5. Column Specifications: Following types:

- Plate number(N)
- Peak asymmetry(As)
- Selectivity value for two different solutes
- Column backpressure
- Retention (k) reproducibility
- Bonded phase concentration
- Column stability

5.1 Plate number

The column plate number (N) is an important characteristic of a column.

N defines the ability of the column to produce sharp, narrow peaks for achieving good resolution of band pairs with small alpha values.

Formula for calculating plate number

$$N = L/H$$

N= number of theoretical plate number

L= length of column

H=height of plate

The plate number should be more than 2000

The column plate number increases with several factors:

1. Well packed columns (column quality)
2. Longer columns
3. Lower flow rates
4. Smaller column packing particles
5. Lowest mobile phase viscosity and higher temperature
6. Minimum extra column effects

Table 1: Plate number for well-packed HPLC columns under optimized conditions.

Particle Diameter(μm)	Column Length(cm)	Plate number N
10	15	6,000-7,000
10	25	8,000-10,000
5	10	7,000-9,000

Tables

5	15	10,000-12,000
5	25	17,000-20,000
5	5	6,000-7,000
3	7.5	9,000-11,000
3	10	12,000-14,000
3	15	17,000-20,000

5.2 Peak asymmetry and peak Tailing

While the column plate number is a useful measure of column quality, peak shape is also equally important.

Peaks with poor symmetry can result in:

- Inaccurate plate number and resolution measurement
- Imprecise quantization

- Degraded resolution and undetected minor bands in the peak tail
- Poor retention reproducibility

Ideally peak asymmetry value is 1 or less than 1.

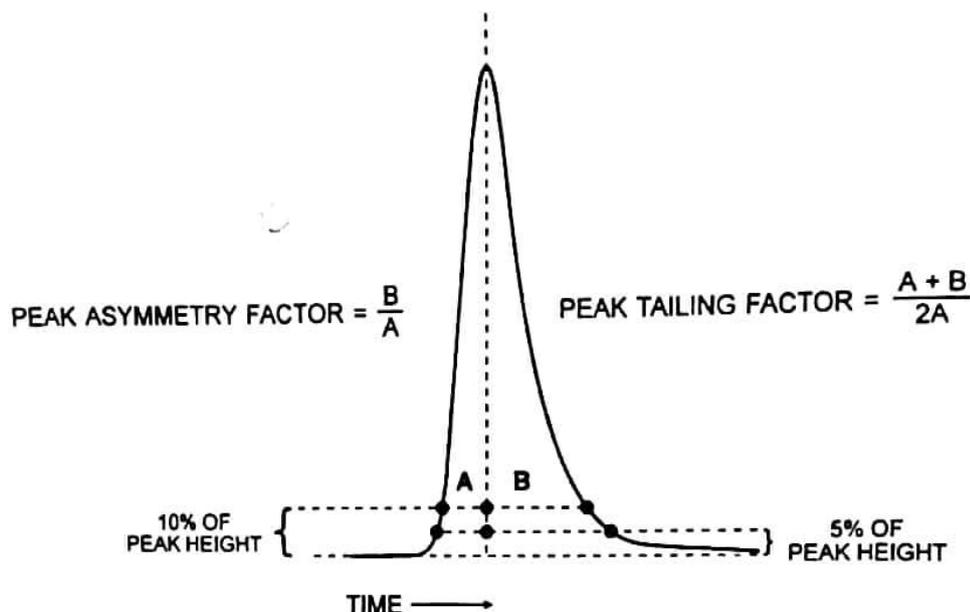


Figure 3: Determine peak Asymmetry and peak tailing factor. In fig. the peak tailing factor calculated at 5% and peak asymmetry factor calculated at 10%.

5.3 Column Failure:How long should a Column Last

The stability and useful lifetime of a well-made column are dependent on how the operator uses and treats the column. All columns are expected to die eventually. A column should be replaced when it no longer provides the performance needed for particular analysis. If the plate number decreases by 50% or resolution falls to

about three- fourths of the original value (e.g. to $R_s = 1.5$ from an initial 2.0 value), a new column may be required.

A column whose performance has degraded somewhat may still be useful for a given assay. $A_s > 1.5$ may also be a sign that the column should be change.

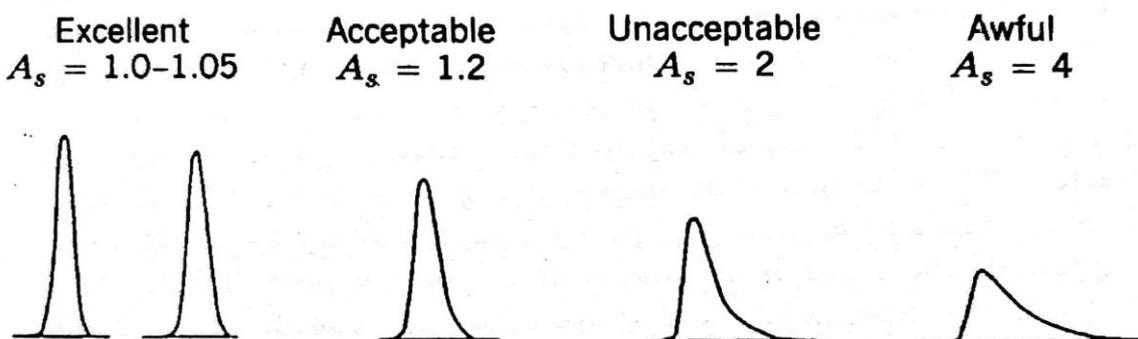


Figure 4: Peak shapes for different asymmetry factor values.

5.4. Retention reproducibility

Long term reproducibility of column from the same manufacturer is an important factor for developing a rugged, repeatable method. Several manufacturer now claim long term reproducibility of their bonded phase

columns. Retention reproducibility calculated by testing system suitability parameter.

Ex. Shows the manufacturing reproducibility for 1 commercial C18 column over a 4 year period.

5.5 Pressure drop

$$P = 3000Ln/to dp^2$$

P- pressure (psi)

L- column length (cm)

n- mobile phase viscosity (Cp) to-column dead time, dp- the particle diameter

New spherical particle-columns should have a pressure drop number not greater than about 30% in excess of that predicted. Columns packed with irregular particles may give higher backpressures.

5.6 Bonded phase Concentration: Well made bonded-phase columns have a dense population of organic groups attached to the surface of the silica support. The actual coverage depends on the size of the organic ligand: high surface concentrations are more difficult to obtain with larger silane groups because of steric hindrance. Columns with densely reacted, sterically protected

groups have a lower concentration of silane groups because of additional steric hindrance by the large protecting groups.

6. Column Problems and Remedies

3 most important kinds of problems in HPLC method development,

- Variability in Retention and Resolution
- Band tailing
- Short column life

6.1 Retention and Resolution Irreproducibility

Reproducible retention and resolution for the peaks in chromatogram are very important when develop routine methods.

Following table summarizes the types of retention and resolution variation that can occur in HPLC.

Table: Retention and Resolution variation in HPLC.

Effect	Causes	Main shanges
Column changes during use	Disturbance in bed Loss of bonded phase Dissolution of silica support Buildup of noneluted material	N k, α N k, N
Extra column effects	From system to system large injection volume; Large tubing volume between injection valve and column and detector; large detector volume; Large volume fitting	N
Poor control of separation	Changes in mobile phase composition, Change in flow rate Change in temperature	k, α Small change in N k, α , small change in N
Slow column equilibration	Insufficient and requilibration time	K
Column overload	Too large a sample mass	k, N
Column-column differencess	Variation in support, bonding	k, α

N- plate number, α - selectivity value, k- retention factor. For basic samples, the retention and reproducibility are minimized by using.

Separation parameters that include

- A less acidic, highly purified silica support
- pH < 3 mobile phase (for reverse phase)

Buffer concentration > 20mM (because of higher solubility and better suppression of unwanted silanol interactions).

If tailing or misshapen peaks occur, the following remedy use:

- Add 30mM triethylamine (for basic compounds) or ammonium acetate (for acidic compounds) to the mobile phase (triethylamine acetate for unknowns)
- If tailing persists, replace the triethylamine with 10mM dimethyloctylamine (or dimethyloctylamine acetate)

- Reduce sample mass to < 1 μ g

Table 3: Remedies for variability in Retention and Reproducibility.

Problem	Causes	Remedies
Poor retention reproducibility	Poorly buffered mobile phases, Selection of the wrong buffer, Too buffer concentration or pH out of the effective range of a buffer	Increase buffer concentration, The pH of mobile phase should be ± 1.5 with pKa of drug
Poor retention reproducibility	Poor control of experimental condition, Changes in mobile phase either during day or from day to day Manually prepared mobile phase	Carefully prepare a new batch of mobile phase and repeat the separation.
ariation in retention	Selective solvent fractionation by evaporation. this effect can occur either during degassing of the mobile phase or on standing Uptake of CO ₂ , which can change the pH of the mobile phase	Minimized by slowly and continuously bubbling helium through the mobile phase reservoir during use, to blanket contents of reservoir, Commercial online solvent degassing often are effective,
Variation in retention	Too large a sample mass can cause retention variation, Column temperature	Less sample mass used, Thermostated column use

6.2. Band tailing

Band tailing are nothing but crosses the acceptance criteria limit i.e. <1.5 .

Peak asymmetry or band tailing can arise from several sources, as summarized in Table.

Table: Causes of Asymmetrical (Tailing) peaks

- Bad column; plugged frit or void
- Buildup of 'garbage' on column inlet
- Sample overload

- Wrong solvent for sample
- Extra-column effects
- Chemical or secondary retention (silanol) effects
- Inadequate or inappropriate buffering
- Contaminating heavy metals

Table 4: Problems and remedies of band tailing.

Problems	Causes	Remedies
Band tailing	Bad column from the manufacturer	Validation done before using
	Void in the inlet of the column and plugged inlet frit	Carefully replacing the frit of the column (without disturbing the column), Reduced by filling the inlet frit void with additional packing
Development of broader tailing peaks	Buildup of strongly retained sample components on the column inlet	Purging the column with a strong solvent. A 20-column volume purge with a mixture of 96% dichloromethane and 4% methanol with 0.1% ammonium hydroxide is often effective for reversed phase columns, Backflushing the column at a low flow rate with a strong solvent may be necessary, Use a guard column
Peak broadening or tailing	Overloading the column	Reducing sample mass injection
	Strong sample injection Injecting the sample in a solvent that is stronger than the mobile phase usually results in early bands that are distorted and tailing When the sample is poorly soluble in the mobile phase, small volumes in a stronger solvent can be injected. However, poorer band shapes, sample precipitation, column blockage and compromised quantitation may result	For poorly soluble material, dissolving the sample in a strong solvent, then diluting with an equal volume of the mobile phase often is successful for sample injection
Tailing and asymmetrical peak	Various chemical effect, Mismatch between the mobile/stationary phase combination and the sample	Using mobile phase and that contain acetate plus triethylamine Changing to entirely different mobile phase – stationary phase combination

6.3 Why do column die

Columns degrade (or 'die') for several reasons:

- Partially blocked (plugged) frit or column bed
- Adsorbed sample impurities
- Initially poorly packed column
- Mechanical or thermal shock creating voids
- Chemical attack on the support or stationary phases

Some symptoms of impending column death are:

- Column backpressure increase
- Tailing bands
- Loss in plate number
- Loss of selectivity
- Retention decrease(*k*)

Table 5: Following table shows the problems and remedies

Why do column die?

Problems	Causes	Remedies
Column frit	<p>Void in the inlet of the column</p> <p>Injections of samples containing particulates ultimately will block the column inlet, reducing normal lifetime of column, particulates also arise from the wear of sample injector and pump seals In-line filters do not eliminate the desirability of removing obvious particulates from the sample</p>	<p>Eliminating by carefully replacing the inlet frit of the column without disturbing the packing. The use of a 0.25 or 0.5 micrometer inlet filter between the injection valve and the column inlet usually eliminate these problems.</p> <p>By filtering or centrifuging the sample before injection, Cloudy samples should be treated with a 0.25micrometer filter paper Changing pump seals and sample valve rotors regularly will minimize problems with frit pluggage</p>
Strongly -held sample components	<p>Buildup of noneluted components is especially a. problem with complex samples such as extracts of biological issues or fluids, oil containing formulations, Improper flushing of column</p>	<p>Reduced by inserting of guard column is a well-packed short length (e.g. 1-2cm) containing a packing equivalent to that in analytical column Flushing the column at least daily with a strong solvent, In extreme cases, the column can be backflushed with a strong solvent, In gradient separations: cleansing column by 100% strong organic solvent periodically at least 20 to 30 column volumes.</p>
Poorly packed columns	<p>Compaction of the packed bed after relatively short use usually results in void in the column inlet and sudden decrease in plate number,</p>	<p>Appropriately column packed</p>
Pressure effects	<p>Sudden pressure surges and any kind of mechanical or thermal shock (should be avoided to minimize changes in peak shape and plate number), Voids can be caused by pressure surges</p>	<p>Special valves are available (e.g. Rheodyne MBB) that avoid the pressure surge of valve switching, Avoid by using well-packed columns and operating at lowest column pressures.</p>
Chemical attack	<p>Stationary/mobile phase combinations that lead to a rapid loss of bonded phase</p>	<p>Use c8and c18 columns usually show good long term stabilith</p>

CONCLUSION

Column play an crucial role in separations of solutes. The lower the particle size (5 μ m, 3 μ m) of stationary phase gives more efficiency of column. The longer the column(25cm) gives more number of theoreticle plate number. Bonded phase silica gives more stability than

others due to attached alkyl groups. more the number of alkyl group more stable, e.g. C₁₈ column. Band tailing reduced by using triethylamine as subset of mobile phase.

Column lifetime increased by column purge neatly by using methanol and acetonitrile solvent, column contamination reduced by using pure samples and stationary phases, use bonded phase stationary phase, maintain pH of mobile phase ± 1.5 with pka of drug etc.

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