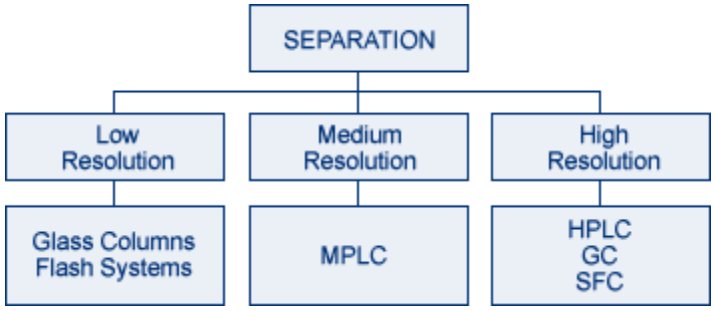
SOP: FLASH CHROMATOGRAPHY



There are a number of different techniques for separation of mixtures and purification of a sample and they vary in their efficiency and resolution. A column consists of two phases a stationary phase (usually silica gel) and a mobile phase (the solvent). Compounds of the mixture interact with the stationary phase based on charge, solubility or adsorption.

Originally Gravity column chromatography was used but Flash chromatography is now a popular method of purification and separation as the solvent is forced to move quicker through the column by applying positive pressure (10 15psi) at the top of the column.

Flash/Gravity chromatography utilises a column filled with a solid support. The most common column packing material is normal phase silica, used with non-polar solvents. The mobile phase or eluent is either a pure solvent or a mixture of different solvents. It is chosen so that the retention factor value of the compound of interest is roughly around 0.2 - 0.3 in order to minimize the time and the amount of eluent to run the chromatography. The eluent has also been chosen so that the different compounds can be separated effectively.

A simple laboratory column runs by gravity flow. The flow rate of such a column can be increased by extending the fresh eluent filled column above the top of the stationary phase or decreased by stop-cock tap control. Faster flow rates can be achieved by using a pump or by using compressed gas (e.g. air, nitrogen, or argon) to push the solvent through the column (flash column chromatography).

The eluent is optimized in small scale pre-tests, often using thin layer chromatography (TLC) with the same stationary phase. The rest of the column is filled with this eluent and with the help of pressure, enables the sample to run through the column and become separated. Flash chromatography used air pressure initially, but today pumps are used to speed up the separation. This technique is considered a low to medium-pressure technique and may be scaled up for separations from a few mg to many tens or hundreds of grams. Flow rates may be from 10ml/min to upwards of 300ml/min.

The particle size of the stationary phase is generally finer in flash column chromatography than in gravity column chromatography. One of the most widely used is silica gel (SiO_2) with a mesh 230 – 400 (40 – 63 µm), while the gravity chromatography requires mesh 70 – 230 (63 – 200 µm) silica gel.

The amount of silica used depends on:

- The Rf difference of the compounds to be separated
- Amount of the sample
- Easy separation uses a ratio of 30:1. Difficult separations need more Silica, which also extends the chromatography time.

SOLVENTS

The first step is to determine the best solvent or solvent mixture. This is done by TLC separation and the aim is to keep the retention factor (Rf) values as low as possible. The chosen solvent must give good separation and move the desired component of interest on TLC analysis to Rf ≈ 0.15 – 0.20. (See SOP: Thin Layer Chromatography (TLC))

Binary (two component) solvent systems – one solvent with a higher polarity than the other are usually best. High polarity solvents increase the rate of elution of compounds. Flash chromatography uses a two solvent mixture – polar & a non-polar component.

Common one component solvents (least to most polar):

1. Hydrocarbons: pentane, petroleum ether, hexane
2. Ether, dichloromethane
3. Ethyl acetate

Common two-component solvents (least to most polar)

4. Ether/Petroleum ether
5. Ether/Hexane
6. Ether/Pentane
7. Ethyl acetate/Hexane– standard solvent mix & best for difficult separations
8. Methanol/Dichloromethane –for polar compounds
9. 10% Ammonia in Methanol/Dichloromethane – can move stubborn amines
10. For basic (i.e. nitrogen containing) compounds, it is sometimes useful or necessary to add a small amount of triethylamine or pyridine to the solvent mixture (about 0.1%).
11. For acidic compounds, a small amount of acetic acid is sometimes useful. In these cases, the acetic acid can often be safely rotavaped away by adding portions of toluene and concentrating to a few ml volumes and repeating this several times. As acetic acid boils at a lower boiling point than toluene, this will remove the acid without exposing the neat compound to it.

Retention in Column Chromatography: Is the measure of the speed at which a substance moves through the column.

TLC data can be used to predict column elution behaviour using the relationship CV = 1/Rf. CV is the number of column volumes required to elute the component from the column regardless of column dimensions. In general, the better the separation, the better the sample load capacity of the column. This is illustrated in the diagram 1, which shows CV vs. Rf as predictors of sample load.

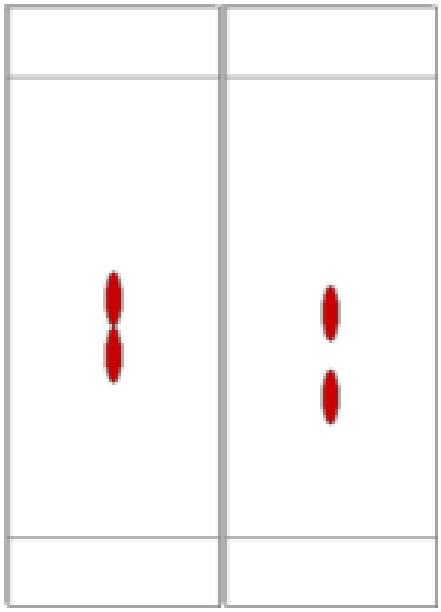
CV plays a greater role in predicting maximum sample loading than Rf.

Unfortunately optimizing a separation using TLC does not necessarily translate to optimum column chromatography. Using a ‘slower’ solvent system can improve the degree of separation. The separate curves in the diagram represent different sample elution concentration profiles over time based on their affinity to the column resin. To calculate resolution, the retention time and curve width are required.

To be successful the column must be uniform in length, diameter and density and the analyte must be concentrated and placed on column is a very narrow band.

TABLE 1 : Solvents vs Solvent Strength for separation.

Solvent	Solvent Strength
Methanol	0.95
Ethanol	0.88
2-Propanol	0.82
Acetonitrile	0.65
Ethyl Acetate	0.58
Tetrahydrofuran	0.57
Acetone	0.56
Dichloromethane	0.42
Chloroform	0.40
Diethyl Ether	0.38
Toluene	0.29
Hexane	0.01



Hexane: EtOAc Hexane: CH_2Cl_2

1:1 1:2

DIAGRAM 1: CV vs. Rf as predictors of sample load and separation.

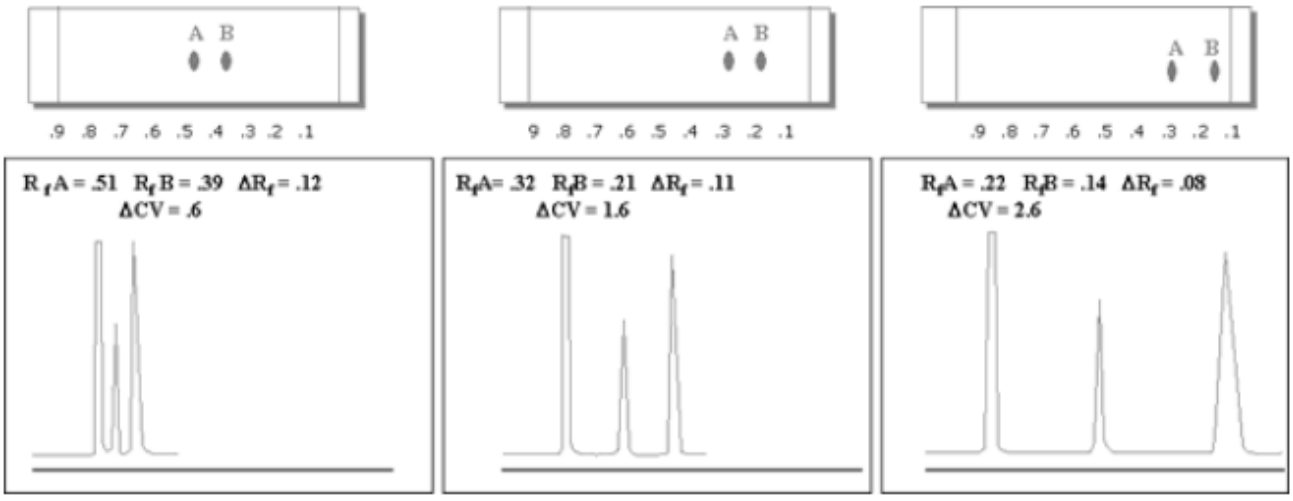


TABLE 2: Common Flash Solvents

Solvent	Density (g/ml)	Elution Strength	Solvent Group	Boiling Point (°C)	UV Cut-off (nm)	TLV (ppm)
n-Hexane	0.66	0.01	1	69	195	100
2 2 4-Trimethylpentane	0.69	0.02	1	99	210	300
Cyclohexane	0.77	0.03	1	81	200	100
1 1 2-Trichloromethane	1.48	0.31	8	61	245	50
Toluene	0.87	0.22	7	110	285	100
Dichloromethane	1.33	0.30	5	40	232	100
Dichloromethane	1.33	0.30	5	40	232	100
Ethyl Acetate	0.90	0.45	6	77	256	400
Methyl-t-butyl ether	0.74	0.48	2	55	210	40
Methyl-t-butyl ether	0.74	0.48	2	55	210	40
Acetone	0.79	0.53	6	56	330	750
Tetrahydrofuran	0.89	0.35	4	66	212	200
Acetonitrile	0.78	0.5	6	82	190	40
Isopropanol	0.79	0.6	3	82	205	400
Ethanol	0.79	0.88	3	78	210	1000
Methanol	0.79	0.70	3	65	205	200
Water	1.00	0.073	8	100	180	-1
Acetic Acid	1.04	0.073	8	118	210	10

PACKING THE COLUMN

To be successful the column must be uniform in length, diameter and density and the analyte must be concentrated and placed on column as a very narrow band.

In fume hood:

1. Usually use a glass column – clamped vertical onto a retort stand
2. Place a glass frit or cotton wool directly above the stopcock – to prevent silica from escaping when opening stopcock during elution.
3. Next place a ≈12mm layer of clean sand above the plug – ensure you fill column to above the taper area
4. Ensure surface is flat – use a rubber tube or cork ring to lightly tap column to obtain this
5. To pack the stationary phase one can use 2 methods: a) Dry or b) Slurry Filling

Dry Filling Set

The Dry Filling Set is employed for filling glass columns with silica gel using compressed gas. Silica gel in the size range of 25 – 200 µm can be packed with this method.

Slurry Filling Set

The Slurry Filling Set is used for wet filling and conditioning of glass columns with silica gel particles smaller than 25 µm.

6. Dry Method:
    - Pour a layer of solvent into column – this will assist in not disturbing the sand
    - Pour 40-63µm dry silica gel ≈125 -150mm high in column via using a funnel
    - Fill column with solvent through a funnel and place it under pressure to rapidly push all the air from the silica gel column – opening/closing stopcock at bottom
7. Slurry method:
    - Prepare slurry by placing silica in beaker and slowly adding solvent to it.
    - Stir gently until homogeneous and there are no air bubbles visible in solvent.
    - Pour 5ml solvent on top of sand
    - Gently pour silica slurry through funnel on top and wait until silica settles down/out– NOT to fast as air bubbles may get trapped.
    - Open stopcock and drain column very slowly to allow silica to settle tapping gently to help remove any air bubbles.
    - Take additional solvent to rinse funnel and walls of column.
    - Drain column of solvent until it is at the top of silica layer – opening/closing stopcock at bottom
    - To drain faster one can place an air pressure to the column. Clip it in place – this will increase the flow rate. Once it nears gel, remove clip and manage flow through manually. It may be necessary to rinse the column one more time to ensure all silica is removed from column walls above the stationary phase.
8. Load sample on top of column with a dropper of Pasteur Pipette- circle the liquid around column wall.
9. To maximise the yield rinse the beaker/test tube which contained the sample and load on to the column.
10. Sample is resting on column. Now to get it into column drain the column open the stopcock until the entire sample is in contact with the stationary phase (column) – this is to ensure when extra mobile phase/solvent is added at the top it is not dissolved the sample.
11. Gently refill top of column first with dropper/pipette until approximately 5ml is added then add rest with solvent from beaker/cylinder.

13. In flash chromatography, one can apply the air pressure to increase flow. The eluate's flow rate is usually 50mm/min.
14. Collect eluent in test tubes (called fractions) which will include the separated compounds as they exit column.
15. Once one test tube is full, change it with new and ensure you arrange the test tube in order of collection sequence.
16. If compound is coloured ensure you collect the whole compound in one fraction.
17. When compound is colourless – one way is to touch the neck of the stopcock – it will feel warm when the compound is eluting.
18. NEVER let the column run dry – keep eye on top of column.
19. Once the first compound eluted one can and the aim is to separate a mixture – it may be necessary to change to another the eluting solvent at the refill point to increase the polarity of the system. This may also dissolve some of the compound and you will have a longer band.
20. Collection test tubes/beakers are changed as soon as compound begins to elute.
21. It is much easier if compound is coloured. If not collect equal size fractions sequentially and label them for further analysis.
22. Once fractions are taken, it is necessary to analyse them to confirm the sample and test for purity.
23. Use TLC to confirm that it is the compound/s you are looking for.
24. Also use TLC analysis on the Colourless compound/s fraction/s to confirm which fraction/s contains the compound/s.
25. Once complete with column run and dry the column completely and clean column.
26. Disconnect column from retort stand and dispose of it in the silica waste disposal container.
27. Invert column and use a rubber tube or cork ring to slightly tap column to eject the Silica gel. One can also rinse the column with a bit of acetone from the outlet of column.

LOADING THE COLUMN

There are two methods to load a column:

1) Wet Method
2) Dry Method

WET METHOD

1) The sample is dissolved in a small amount of solvent.
2) Gently add a narrow band of the concentrated sample with a transfer/Pasteur pipette on top of the column.
3) Sometimes the solvent choice is more polar than the eluent solvent used in the column, but only a few drops of sample/solvent solution is loaded otherwise it can interfere with the eluent and purification/separation.
4) In the latter it may be necessary to rather use the dry method.
5) Use a transfer pipette to place sample gently and slowly on top of column. DO NOT disturb column.

DRY METHOD

1) Dissolve the sample in a very small amount of solvent and add 100mg silica.
2) Let the solvent evaporate until only a dry powder is left.
3) Place the dry powder on top of the prepared column.
4) Add fresh eluting solvent on the top in order for the elution to begin.

DIAGRAM 2: Column Packing and Settling Procedure.

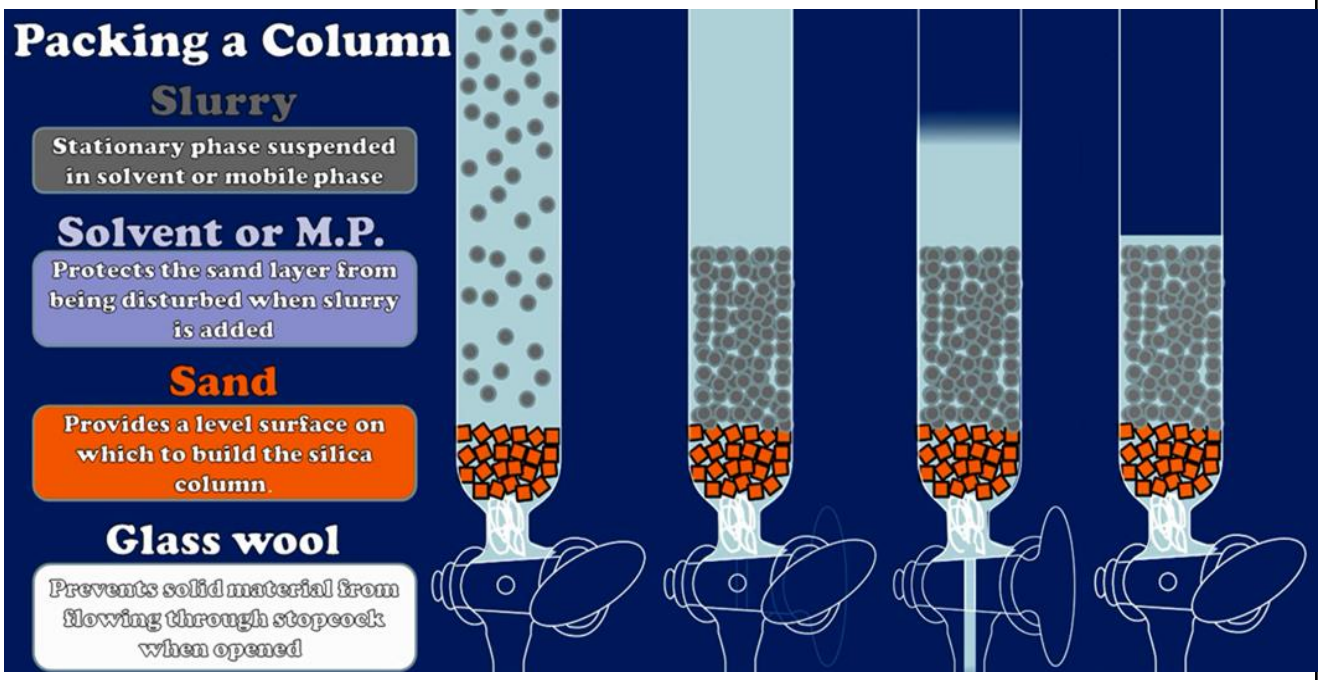


DIAGRAM 3: Sample Loading and Separation Procedure.

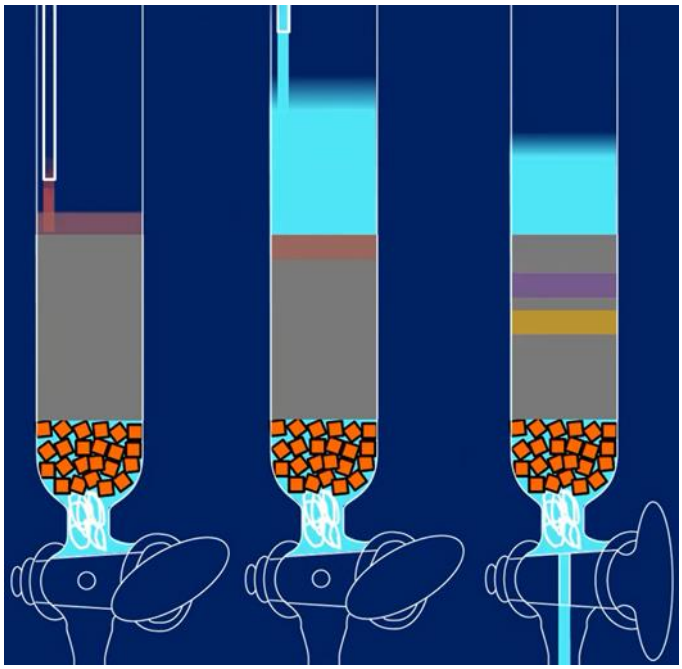


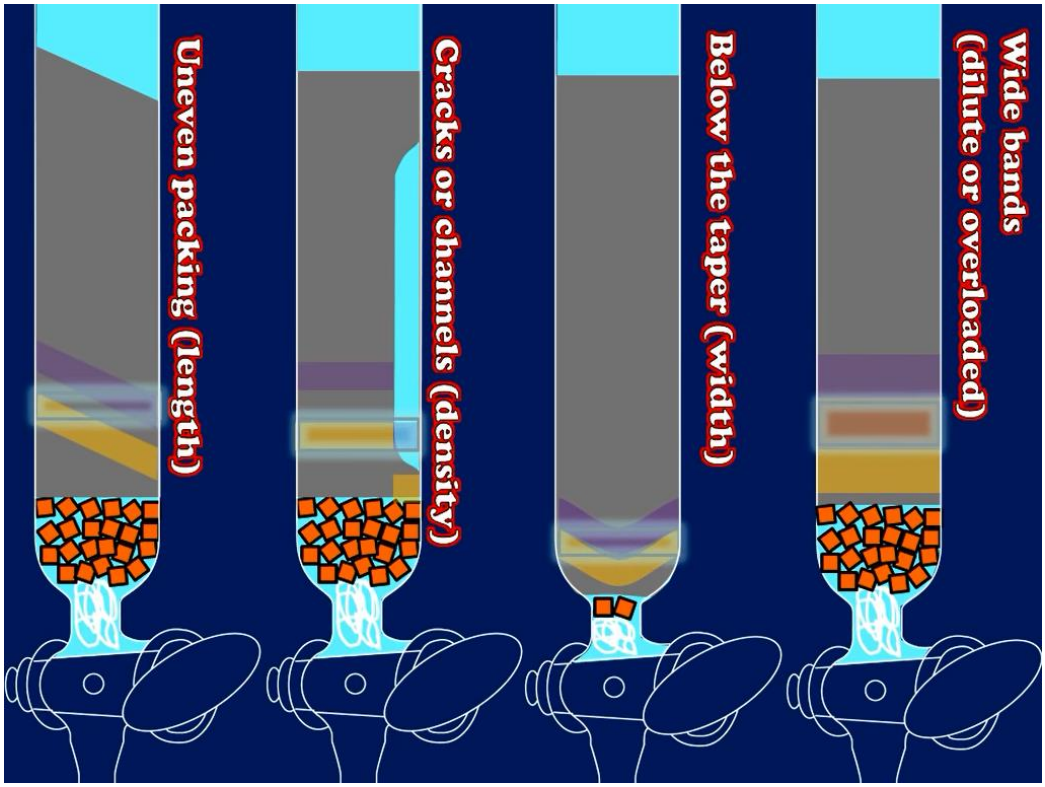
TABLE 3: Typical Volume of Eluent Required for Packing and Elution of Column.

Column Diameter (mm)	Volume of eluent* (ml)	Sample Load (mg) Rf> 0.2	Rf>0.1	Fraction Size (ml)
10	100	100	40	5
20	200	400	160	10
30	400	900	360	20
40	600	1600	600	30
50	1000	2500	1000	50

* Typical Volume required for equilibrium of the column and elution.

ISSUES

1) Cotton wool must not be placed in too tightly only snug.
2) Sand must be above taper of column. One can place another layer of sand on top of column. Ensure it is level and rinse off column walls with solvent. This ensures that the silica is not disturbed when loading the sample.
3) Once some solvent is loaded on top of sand at top of column, open stopcock to ensure it works and there is a flow of liquid. REMEMBER to close it again.
4) Column must NEVER run dry – look regularly at meniscus of solvent at top of column - during preparation or actual process. The solvent must always be above the silica gel.
5) Column must at all times be kept vertical.
6) One can re-use the pure solvent used to pack and wash column and walls.
7) Use silica tubing or cork ring to lightly tap column to assist silica to settle better and to get it even.
8) A less polar compound will exit column first and more polar compound last.
9) Incorrectly packed column causes the following problems indicated in diagram below:



HEALTH & SAFETY ISSUES

1) Always work in a fume hood as solvents are usually very flammable and may cause health effects if breathed in.
2) Ensure there are no heat sources close to column or fractions as it may set alight.
3) Silica causes health problems such as silicosis, thus do not breathe it in.
4) Always cap chemicals immediately after use.
5) Ensure Silica waste is discarded of in the Silica waste container.