A Roadmap to Successful Flash Chromatography

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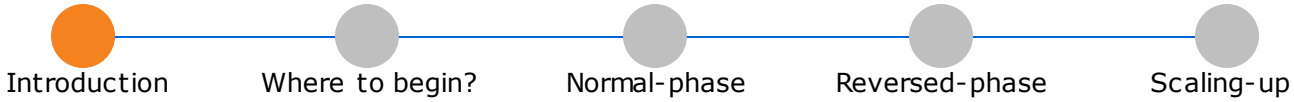
Biotage®

Outline

- Introduction to flash chromatography
- Where to begin?
- Normal-phase flash chromatography
  - Method development and optimization
  - Sample loading options
- Reversed-phase flash chromatography
  - Method development and optimization
  - Lipophilic compounds
  - Ionic/ionizable compounds
- Scaling-up

Flash Chromatography
Definition



What is flash chromatography?

- Flash chromatography is a preparative liquid chromatography purification technology that separates and isolates one or more compounds from a mixture of compounds

How does it work?

- It works by using...
  - A solid sorbent or media (stationary phase) to attract the sample's compounds
  - A solvent or solvent blend (mobile phase) to *selectively* elute the compounds from the stationary phase

Flash Chromatography
Lexicon

Basic terminology

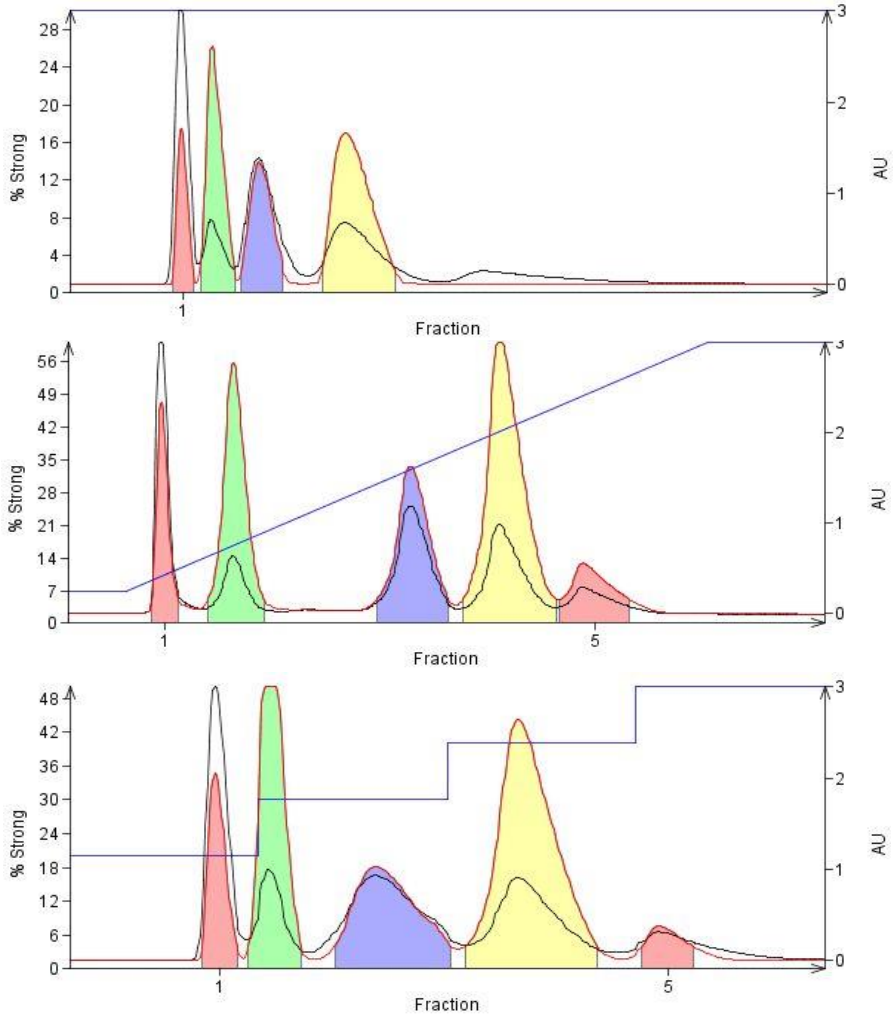
- **TLC** = Thin-layer chromatography. Used to evaluate solvent mixtures for flash method optimization
- **Rf** = Retardation factor. A measure of a compound's elution on a TLC plate relative to the solvent migration. Used to help determine how much solvent is required to elute the compound (expressed in CV)
  - Rf = D_1 / D_{SF}
  - Scale is 0-1
- **CV** = Column volume. The volume of a packed flash column or cartridge specified in mL. Also used as a measure of compound retention, CV = 1/Rf
- **ΔCV** = Delta CV. The difference in elution volumes for two adjacent compounds. This value is used to determine loading capacity for a mixture of compounds
- **Selectivity**. The amount of separation between adjacent eluting compounds
- **Isocratic**. A mobile phase in which the solvent ratio remains constant throughout the purification
- **Gradient.** A mobile phase that changes polarity (a.k.a. strength) over the course of the purification moving from a weaker solvent blend to a stronger solvent blend. There are two gradient types commonly used
  - Linear gradient. A gradient that is created at a constant rate (% strong solvent/CV)
  - Step gradient. A gradient created by increasing solvent strength in a step-wise fashion.
- **Equilibration**. The process used to prepare the flash column for purification. Equilibration displaces the air in a new column and conditions the stationary phase to a constant polarity. Typically, 3 to 5 CV are required for full equilibration
- **Dry load**. A sample introduction technique in which the sample is dried onto a sorbent prior to putting inline with the flash column. The typical benefit is improved separations and better fraction purity
- **Flow rate**. The solvent pumping rate in mL/min
- **Linear velocity.** Solvent flow expressed in distance per unit of time (mm/sec or cm/min). Used when scaling up a purification to ensure a consistent sample/sorbent interaction rate (kinetics)

Flash Chromatography
Elution Methods

- **Isocratic**
  - Two solvents are mixed at a specific ratio (based on TLC) and kept at that ratio during elution
  - Compound elution typically matches TLC prediction based on the relationship CV=1/Rf

- **Linear gradient**
  - Two independent, miscible solvents are pumped and blended at continually changing ratios prior to reaching the cartridge
  - Provides better peak shape, improved resolution, improved loading capacity, and higher concentration fractions

- **Step gradient**
  - Two independent, miscible solvents are pumped and blended step-wise at increasingly stronger ratios prior to reaching the cartridge
  - Can provide faster elution, increased sample loading, enhanced selectivity, improved resolution, higher concentration fractions, and reduced solvent consumption



Flash Chromatography
Operation Modes

Normal-phase

- Polar stationary phase
  - Silica
  - Polar bonded
    - NH
    - Diol
    - CN
- Non-polar solvents
  - Hexane/heptane
  - Ethyl acetate/acetone
  - DCM
  - Alcohols
  - Ether
  - Toluene

Reversed-phase

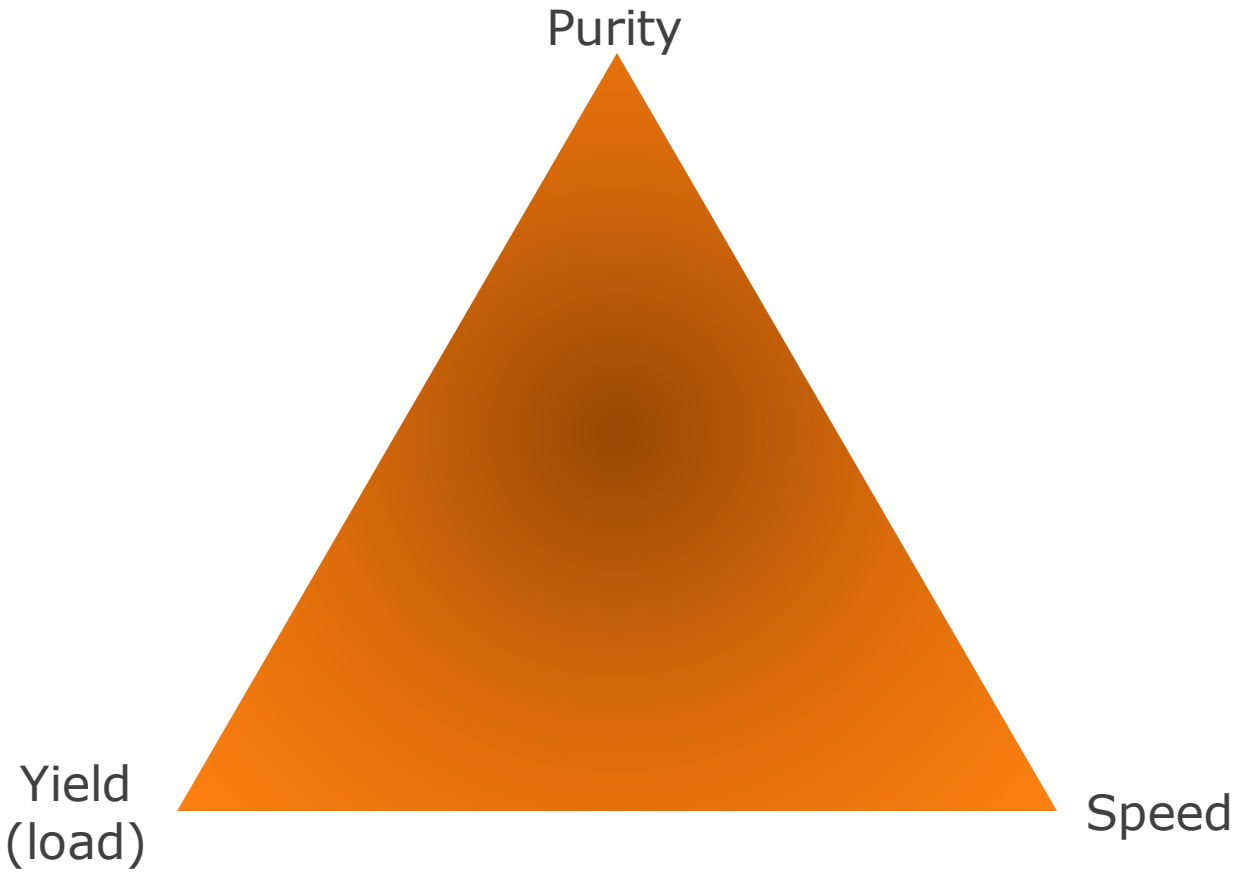
- Non-polar stationary phase
  - C18
  - C4
- Polar solvents
  - Water
  - Methanol/ethanol
  - Acetonitrile
  - Acetone
  - Tetrahydrofuran (THF)

Remember, for normal- and reversed-phase…

Like Likes Like

Where to Begin?
Understand your Purification Goals

Introduction | Where to begin? | Normal-phase | Reversed-phase | Scaling-up



Where to Begin?
Understanding Your Sample

- Only C and H
  - Organic soluble compounds
  - Reversed-phase
  - Normal-phase
- Organic amines
  - Organic soluble
    - Amine-bonded silica
  - Water soluble
    - Reversed-phase
- Most others
  - Normal-phase silica (if organic soluble)
  - Reversed-phase
    - Water soluble or soluble in higher polarity solvents
    - Ionic/ionizable
    - Acidic/basic

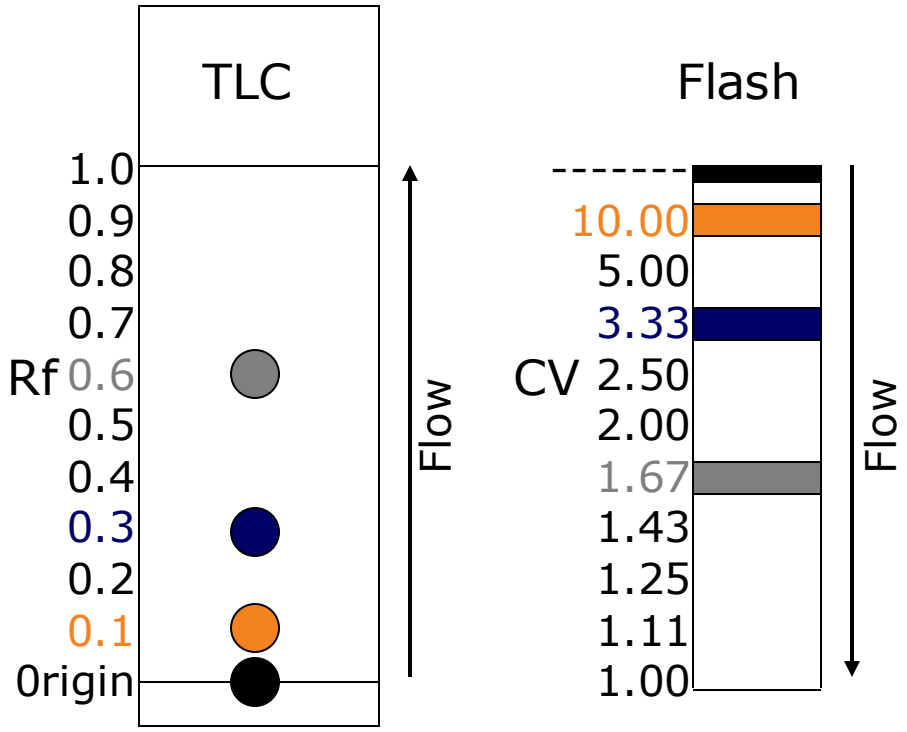
Where to Begin?
Understanding Your Sample - Solubility

Rank	Solvent polarity	Mode
1	Dimethyl sulfoxide (DMSO)	Reversed-phase
2	Dimethyl formamide (DMF)	Reversed-phase
3	Water	Reversed-phase
4	Methanol (MeOH)	Reversed-phase
5	Acetonitrile (MeCN)	Reversed-phase
6	Ethanol (EtOH)	Reversed-phase
7	Propanol (PrOH)	Normal-phase*/Reversed-phase*
8	Acetone	Normal-phase*/Reversed-phase*
9	Tetrahydrofuran (THF)	Normal-phase*/Reversed-phase*
10	Ethyl acetate (EtOAc)	Normal-phase*/Reversed-phase*
11	Ether	Normal-phase/Reversed-phase
12	Methylene chloride (DCM)	Normal-phase/Reversed-phase*
13	Toluene	Normal-phase/Reversed-phase*
14	Hexane	Normal-phase/Reversed-phase*

* Dry loading is best for these solvents

Method Development
Normal-phase

Introduction | Where to begin? | Normal-phase | Reversed-phase | Scaling-up



- Compound retardation factor (Rf)
  - Inversely proportional to flash elution volume (CV) in isocratic elution mode
    - CV = 1/Rf
  - ΔCV used to determine sample load

Method Development

Normal-phase Elution Solvents

Solvent	Group	Strength
Methanol	II	0.70
Ethanol	II	0.65
Propanol	II	0.60
Tetrahydrofuran	III	0.53
Acetone	VIa	0.50
Acetonitrile	VIb	0.50
Ethyl acetate	VIa	0.43
Ether	I	0.40
Dichloromethane	V	0.32
Toluene	VII	0.22
Heptane	---	0.01
Hexane	---	0.01

- Scout solvents from different selectivity groups
  - Maximize separation (ΔCV)
- Adjust solvent strength to elute target within the Rf range of 0.15 to 0.4

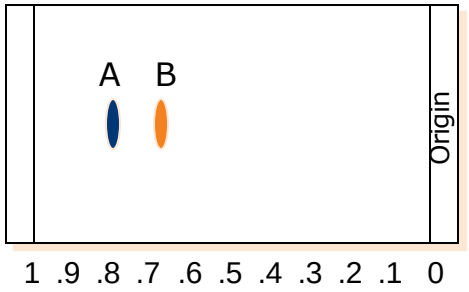
Method Development

Normal-phase

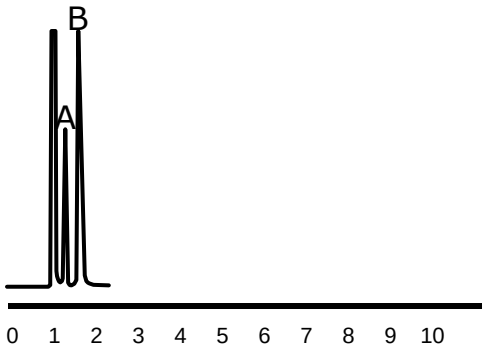
	Rf	ΔRf	CV	ΔCV
	0.10		10.0	
Optimal Range	0.15	0.05	6.7	1.7
	0.20	0.05	5.0	1.0
	0.25	0.05	4.0	0.7
	0.30	0.05	3.3	0.5
	0.35	0.05	2.8	0.3
	0.40		2.5	
	0.45		2.2	
	0.50		2.0	
	0.60		1.6	
	0.70		1.4	
	0.80		1.25	
	0.90		1.11	
	1.00		1.0	

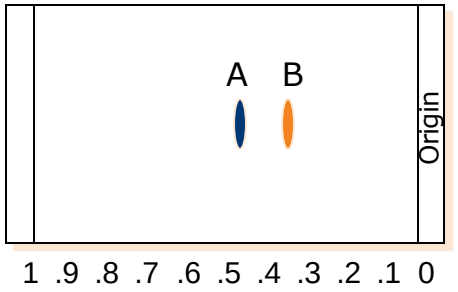
Rf – CV relationship

ΔRf or ΔCV?

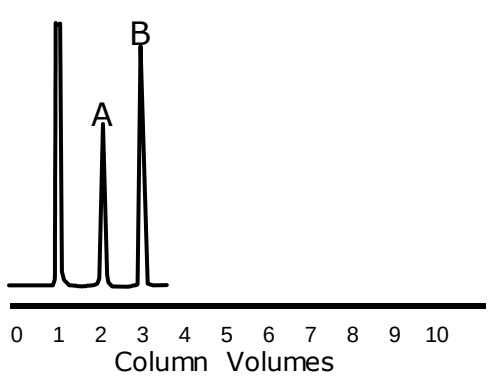


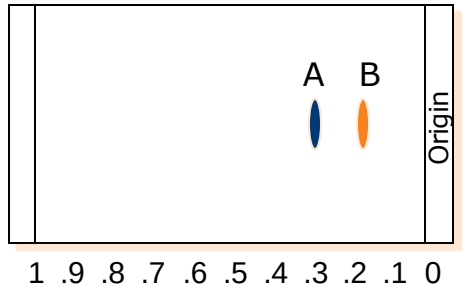
R_fA = .80 R_fB = .67 ΔR_f = .13
ΔCV = 0.24



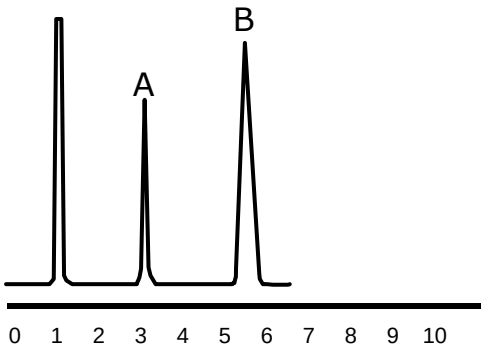


R_fA= .47 R_fB = .34 ΔR_f = .13
ΔCV = 0.81





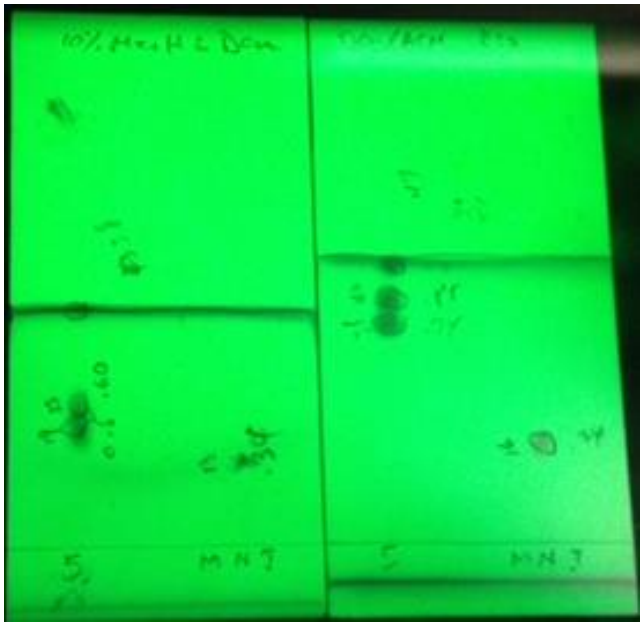
R_fA = .32 R_fB = .18 ΔR_f = .14
ΔCV = 2.43

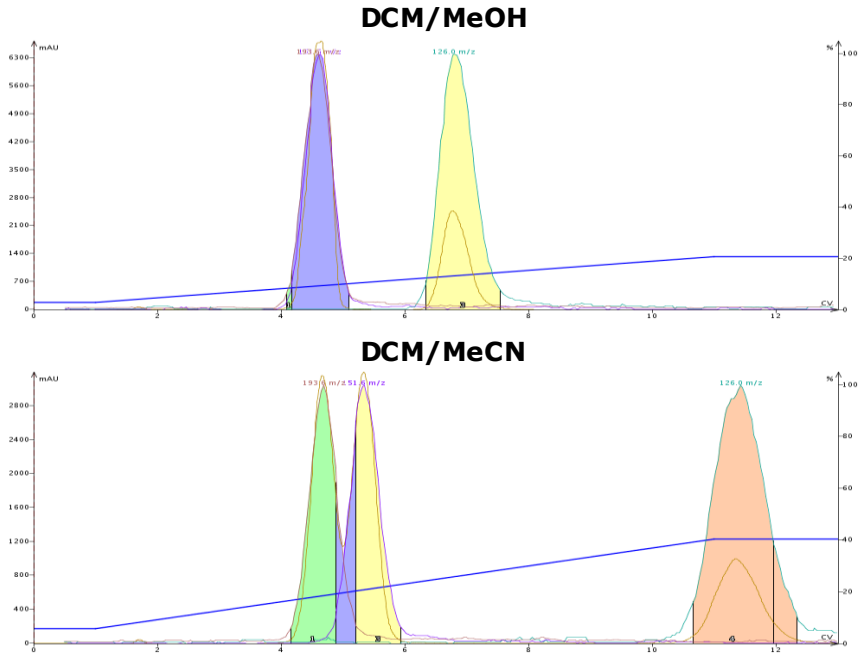


- ΔRf constant
- Increasing ΔCV with decreasing Rf
- Predict maximum sample loading with ΔCV, not ΔRf

Solvent Choice Impact

Solvent Chemistry Matters





Goals and Chemistry

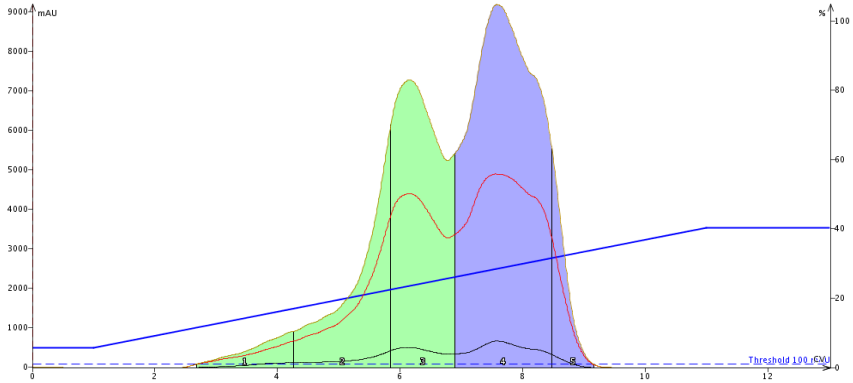
- Goal
  - Separate and isolate each compound
- Solubility
  - Alcohol
  - Acetone
- Chemistry
  - Contains carbon, hydrogen, and oxygen
    - Hydroxyl groups
    - Carbonyls

Method Development and Chromatography

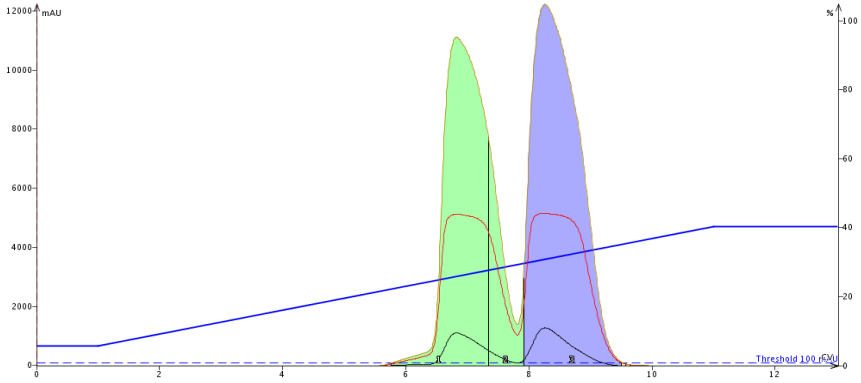
- TLC
  - DCM/MeOH (9:1)
  - DCM/MeCN (8:2)
  - Same solvent strength (0.36)
- Flash
  - Liquid load in acetone (150 mg/0.15 mL)
  - DCM/MeOH unable to separate two compounds
  - DCM/ACN successful

Loading Techniques
Liquid or Dry?

1-mL liquid load (acetone), 200 mg sample, 10 gram Biotage® SNAP Ultra, hexane/EtOAc gradient



Dry load (1 gram silica), 200 mg sample, 10 gram Biotage® SNAP Ultra, hexane/EtOAc gradient



Liquid Loading

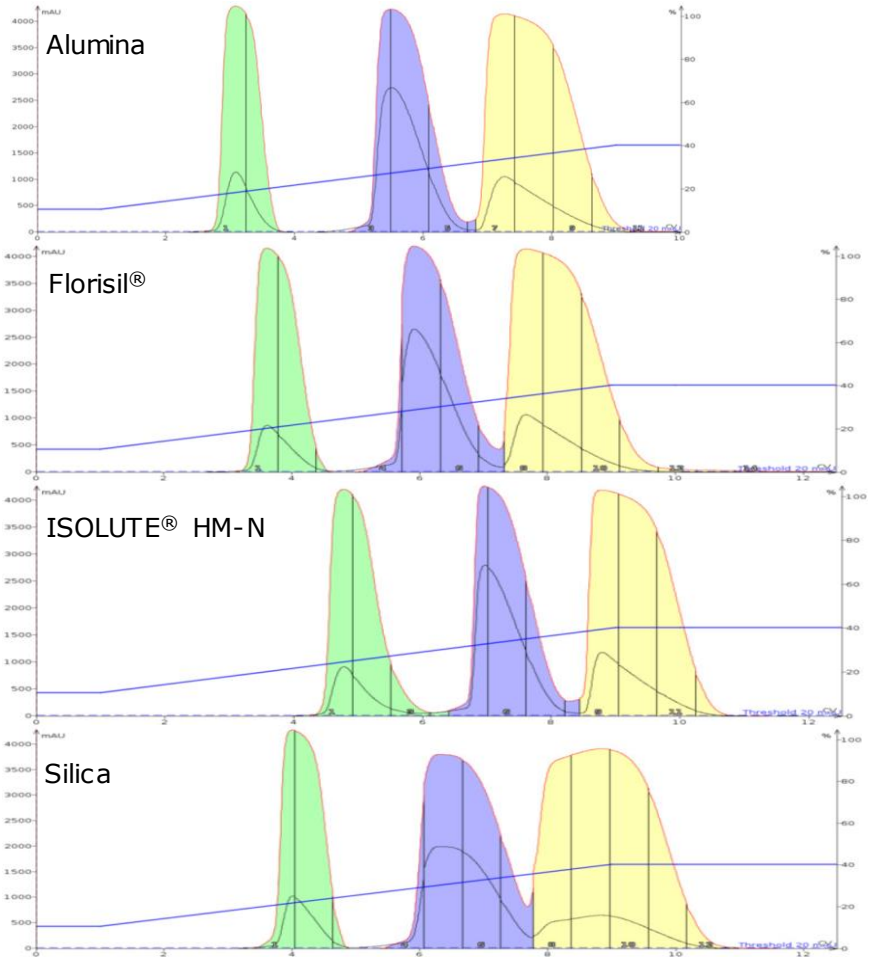
- Weak dissolution solvent
- High sample concentration
  - >1 g/mL
- Low load volume
  - <1% of a CV

Dry Loading

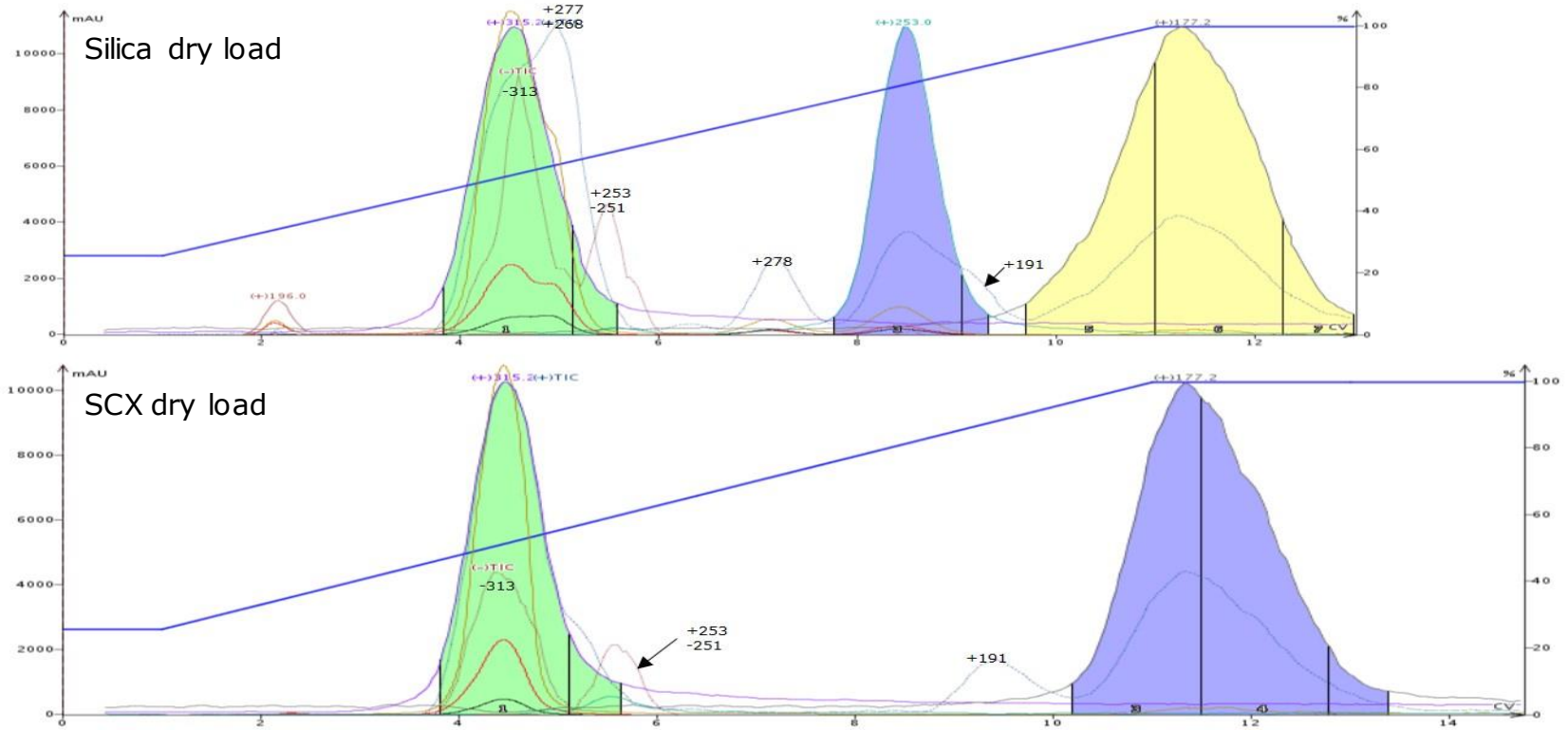
- Solvent dissolves sample completely
- Solvent is volatile
- Mix with sorbent and dry completely

Dry Loading
Options and Techniques

- Select a sorbent
  - Silica
  - Diatomaceous earth (ISOLUTE® HM-N, Celite)
  - Alumina
  - Florisil®
  - Ion exchanger (silica-based)
  - C18
- Weigh enough sorbent
  - Good sample to sorbent ratios range between 1:2 to 1:4
- Dissolve sample in minimal volume of volatile solvent and mix with sorbent
- Dry completely
  - Free-flowing powder
- Use empty Samplet® cartridge or dry load vessel (DLV)



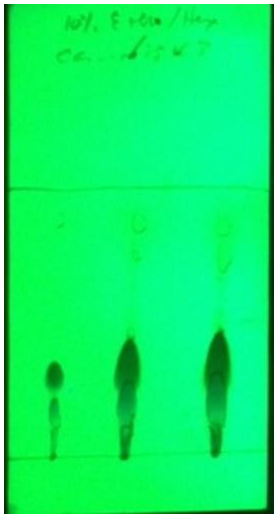
Dry Loading
Options and Techniques



- Impurity scavenging
  - Remove interfering compounds using a scavenger such as an ion exchange media

Flash Chromatography in Practice

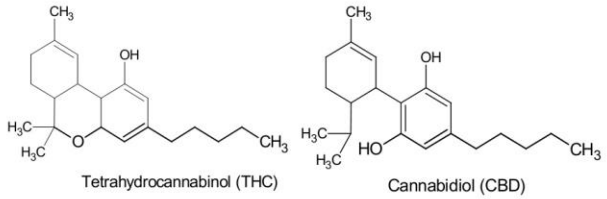
Natural Product Purification - Cannabis Extract

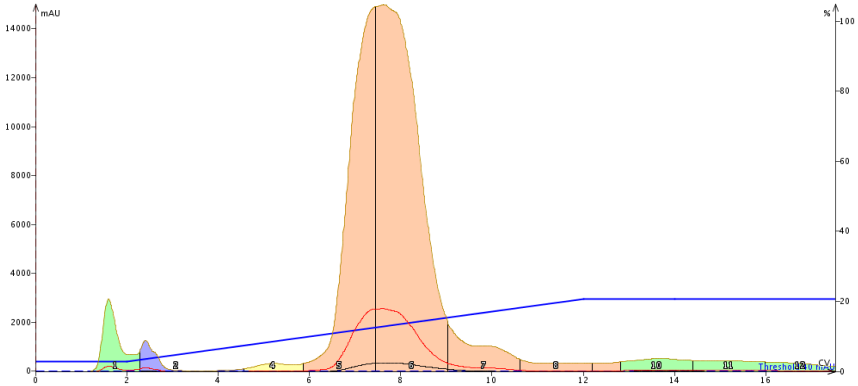


Waxes, terpenes, carotenoids

Cannabinoids

Cannabinoid acids
Chlorophyll and other polars





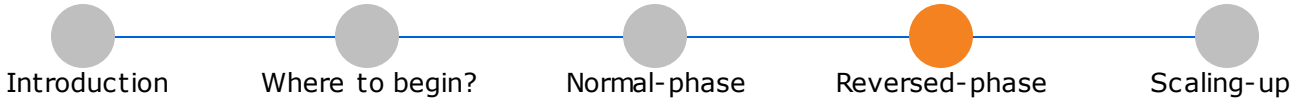
Goals and Sample Chemistry

- Goals
  - Clean-up extract to isolate THC + related compounds from undesirable compounds
- Chemistry
  - Contains carbon, hydrogen, oxygen
  - Hydroxyl groups
  - Solubility (hexane)

Method Development and Chromatography

- TLC
  - 10% ether in hexane
- Flash
  - Biotage® SNAP Ultra, 10 g
  - Liquid load
    - 250 mg (0.1-mL hexane)

Method Development
Reversed-phase



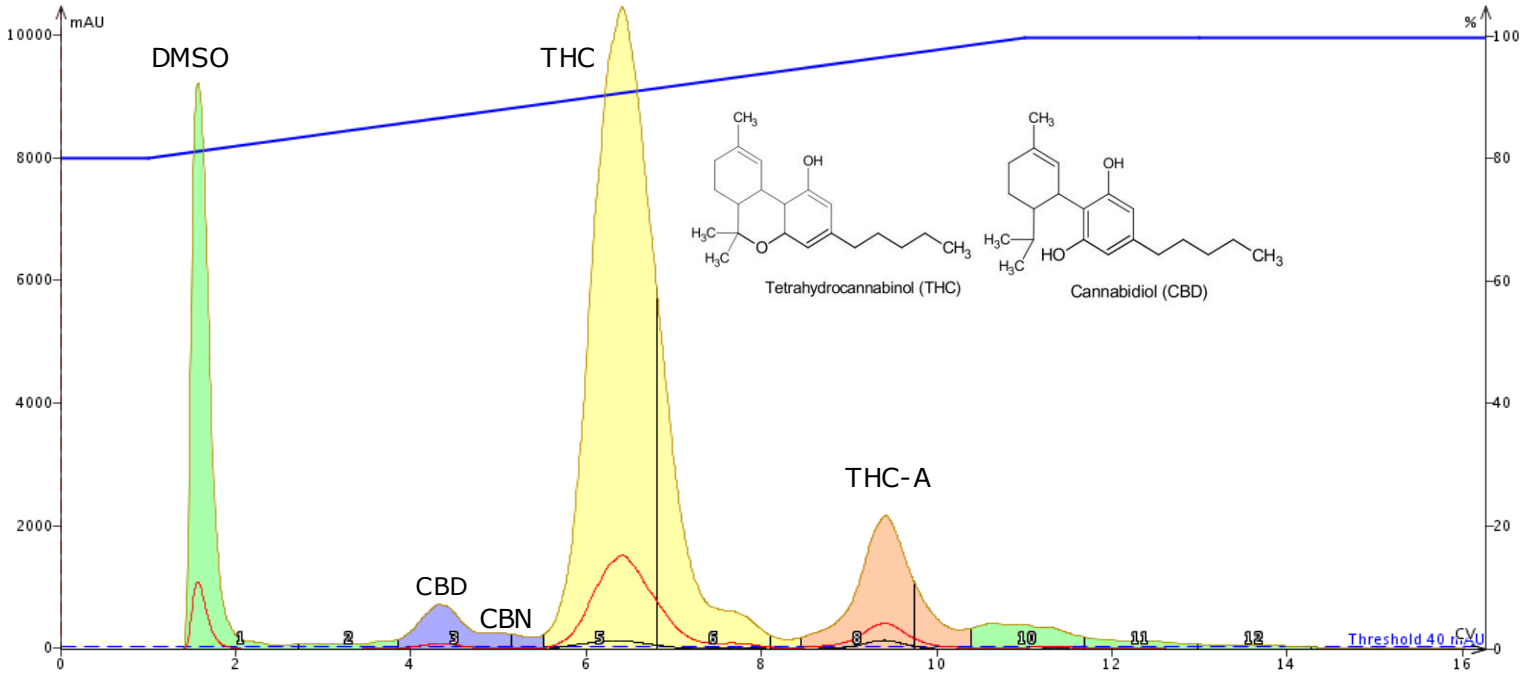
- Use HPLC
  - Use a scaling column
    - HPLC column packed with reversed-phase flash sorbent
  - Adjust solvents and gradient to optimize separation
    - Water/methanol
    - Water/acetonitrile
    - Acetone and THF can be used in place of methanol and acetonitrile for more hydrophobic compounds
  - Add volatile acid, base, or buffer as needed
    - Improve peak shape
    - Improve separation

Method Development
Reversed-phase

- Sample solubility determines starting conditions
  - Water, DMSO, DMF soluble
    - Start gradient at 5% B for 1 CV
      - B is usually methanol or acetonitrile
    - Finish gradient at 50% B (over 10 CV)
  - Methanol, acetonitrile, or other organic solvent soluble
    - Start gradient a 50% B for 1 CV
    - Finish gradient at 100% B (over 10 CV)
- If compound of interest elutes too early, reduce final % B
- If target elutes too late, increase starting % B
- If target does not separate from impurities change solvent B, modify pH/ionic strength, or change gradient slope

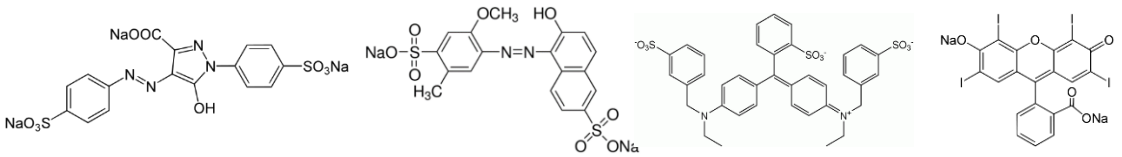
Flash Chromatography
Cannabis Extract

Methanol/water gradient

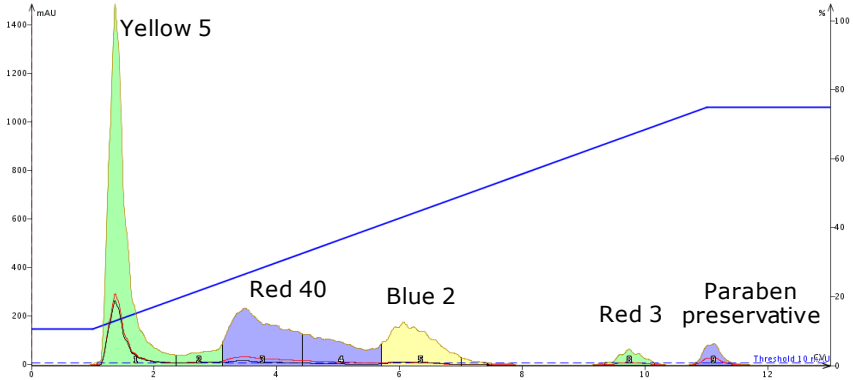


- Goal
  - Isolate CBD, CBN, THC, other components
- Solubility
  - Hexane
  - Alcohols
  - DMSO
- Chemistry
  - Contains carbon, hydrogen, oxygen
    - Hydroxyl groups
    - Ether
- Flash
  - Biotage® SNAP Ultra C18, 12 gram
  - High % organic solvent
  - 50 mg load, DMSO

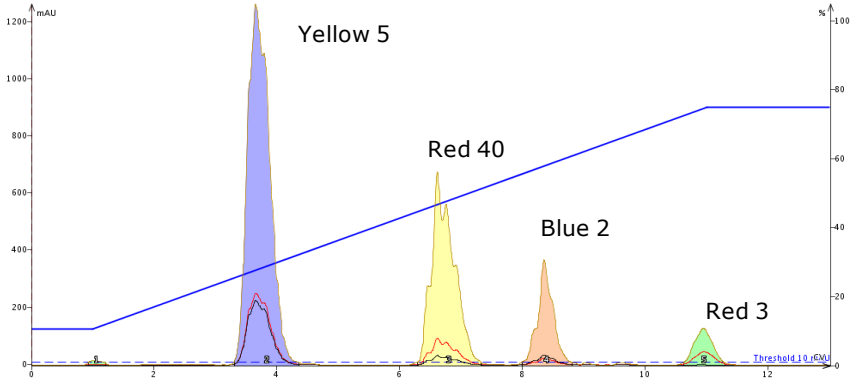
Flash Chromatography
Ionic, Water-soluble Compounds



Methanol/water gradient

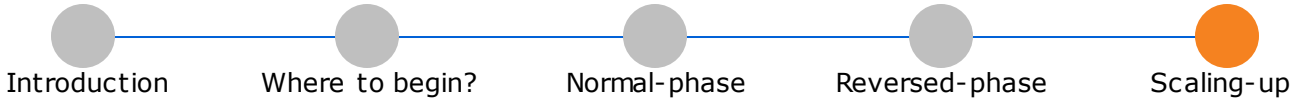


Methanol/water (with 0.1M ammonium acetate, pH 7) gradient



- Goal
  - Separate and isolate each dye
- Solubility
  - Water
  - Alcohol
- Chemistry
  - Ionic/ionizable
  - Carboxylic acid groups
  - Sulfonic acid groups
  - Quaternary amine

- Flash
  - Biotage® SNAP Ultra C18, 12 gram
  - Low organic solvent
  - Un-buffered mobile phase
    - Low retention
    - Broad peaks
  - Buffered mobile phase
    - Increased retention
    - Sharp peaks

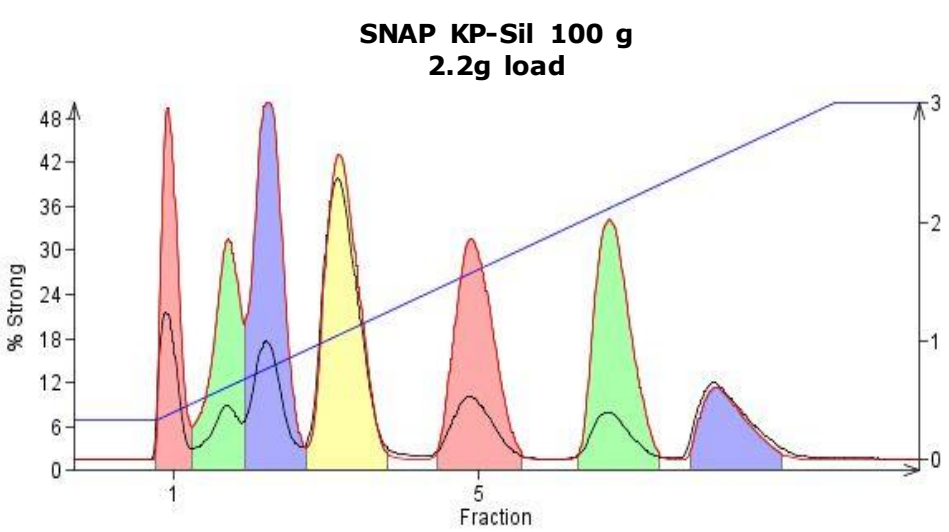


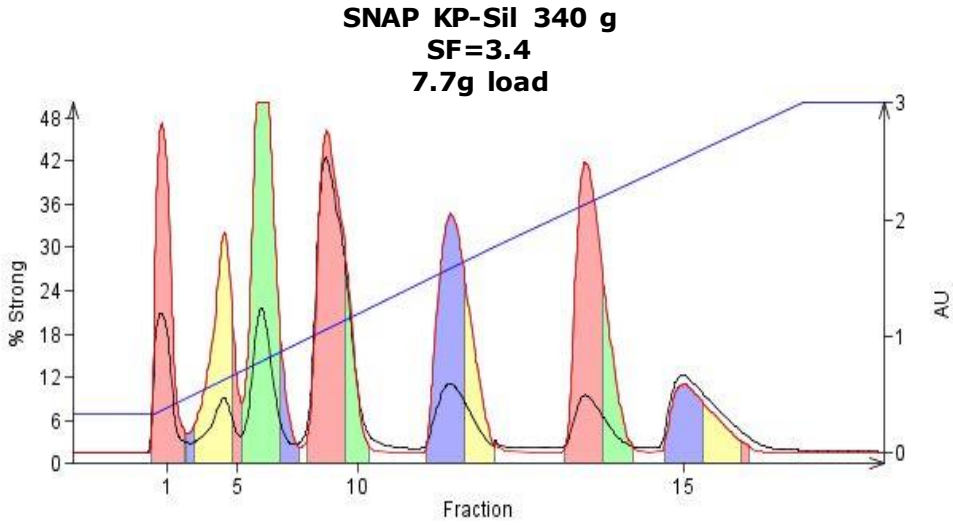
Milligrams to grams to kilograms

- Small-scale flash easily scaled
  - Use scale-up factor (SF)
  - Multiply SF times small-scale load to get large scale load

- Scale factor equal to the column volume or media mass ratio
  - Media must be identical
  - SF = CV_L / CV_S
  - SF = Large column grams/Small column grams

Flash Chromatography
Scaling up





- If a 10 gram cartridge purified 100 mg, then a 100 gram cartridge will purify a gram and a 1000 gram cartridge will purify 10 grams
  - Stationary phase must be identical
- No method modification required other than flow rate and rack size
  - Flow rate does not scale!
  - Need to match linear velocity

Flash Chromatography Success
Summary

- Flash chromatography
  - Determine purification goal(s)
  - Determine crude sample solubility
    - Liquid or dry load
  - Understand possible functional group impact on the purification
    - Normal- or reversed-phase
    - Neutral or modified solvents
  - Spend a little time on method development