

An online tutorial for the use of chemical analysis in the study of biological interactions

(Adapted from Dr Tom Prescott, RBG Kew)

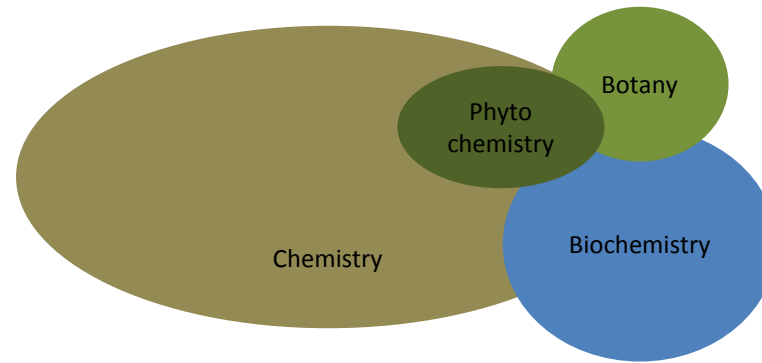
An online tutorial for students intending to incorporate chemical analysis into their work on biological interactions at NRI

In biological interactions we carry out a variety of techniques that allow us to analyse plant extracts and determine the chemical structures of their components. There are several reasons for doing this:

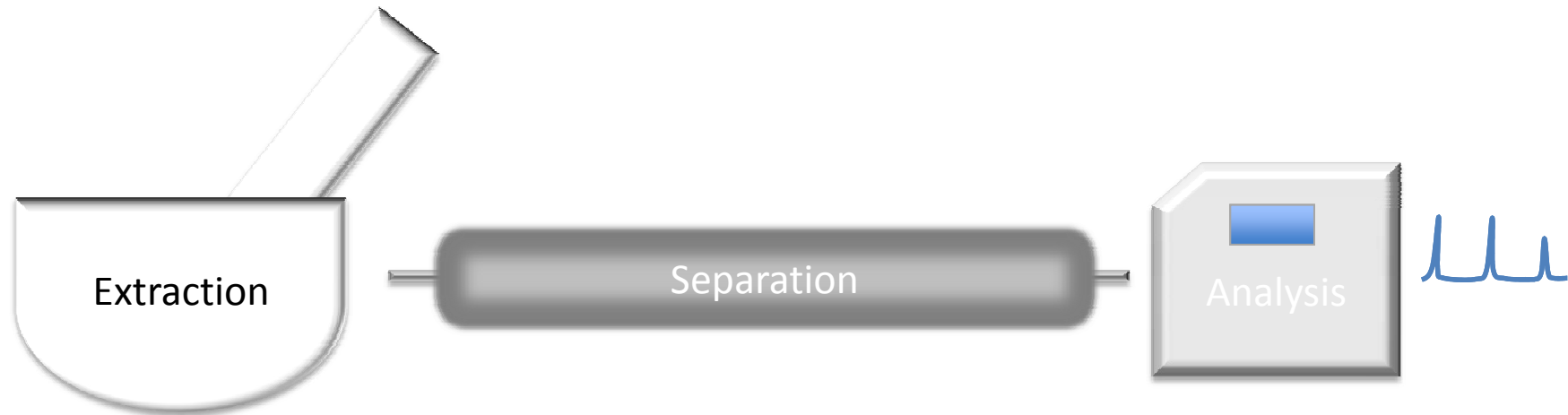
- 1.To gain an understanding of the secondary metabolites in a given plant species and determine if they are novel
- 2.To provide information to help resolve taxonomic questions that cannot be solved using standard botanical methods (chemotaxonomy)
- 3.To determine if plant material contains specific toxic or legally prohibited compounds for forensic purposes
- 4.To search for compounds with useful biological properties e.g. compounds with pesticidal, medicinal or cosmetic properties.

Below are links to online reading and videos that will help you to gain an understanding of the principles behind the techniques we use. Follow the links and then conduct your own research for each item to gain as full an understanding as possible before you start work in the lab.

Conceptual overview



Phytochemistry and related disciplines



The phytochemical process

Before you start work in the lab you should know

- What sort of work goes on in the lab and why
- Basic health and safety
- How to use a Gilson pipette
- How to perform concentration dilution calculations
- Have an insight into the types of phytochemical compounds commonly encountered in the lab
- Understand the principles of plant extraction
- Understand the concept of hydrophobicity and the difference between polar compounds and apolar compounds
- Understand what chemical characteristics determine a compounds interaction with a stationary phase
- Understand the principles of how and why a compound is isolated
- Understand the principles of column chromatography (stationary phase, mobile phase, solvent gradients, gel filtration hydrophobic interaction).
- Understand the principles behind HPLC and why it is necessary, what are its advantages and disadvantages, how are HPLC peaks formed
- Understand that some compound classes have characteristic absorption spectra which can be detected on an HPLC
- Understand what thin layer chromatography is and why its used
- Understand the basic concept of LC-MS
- Understand the basic concept of NMR

Do's and don'ts in the lab

- Adhere to all relevant safety measures and procedures
- Plant extracts may contain toxic compounds, wear gloves and a lab coat to reduce risk of contact with skin
- Some lab consumables are very expensive (C-18 and LH-20 column sorbents) always ask before use and always re-use where possible
- Always properly close fridge-freezer and cold room doors after use
- Always label your lab materials (bottles etc) and store or dispose of them appropriately before you leave
- When weighing out compounds or plant material always tidy up after yourself by putting chemicals back on shelves where you found them and cleaning the balance after using it
- Know how to dispose of organic solvents safely, (in the fume cupboard there is a non-chlorinated and chlorinated solvent waste container)
- Never contaminate stock chemicals by putting used spatulas or pipette tips into containers
- Measure out solvents by pouring them into a glass beaker do not pour directly
- Never over wind pipettes beyond their maximum volume
- Don't try to force open fridges that are pressure sealed, instead wait for pressure to equalise

How to use a Gilson pipette

1L = 1000ml
1ml = 1000 μ l
0.1ml = 100 μ l



http://www.youtube.com/watch?v=uEy_NGDfo_8

Weighing compounds



Use the balance on the left to weigh out gram quantities, use the one on the right to weigh out mg quantities. How many mg does the right hand balance read?

Making solutions of known molarity

Q. You have 3.24mg of a compound of molecular mass 423. How do you make a 1mM stock solution, and then make 400 μ l of a 370 μ M solution

A. Dissolve 3.24mg in 7.66ml solvent to make a 1mM stock solution, $(3.24/423 \times 1000)$ then take 148 μ l and dilute with 252 μ l to make a 370 μ M solution (desired conc/present conc x desired final volume)

What pipettes and tips would you use to do this?

<http://chemistry.about.com/od/lecturenotes13/a/concentration.htm>



Sample concentration

You have an extract or fraction, how do you concentrate it? Research these sample concentration methods and find out their advantages and disadvantages

Heating block



Rotary-evaporator

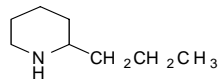


Freeze-dryer

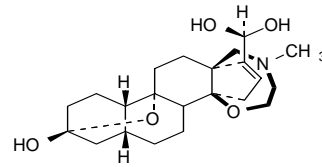
<http://www.youtube.com/watch?v=dKQldSDso5E>

Introduction to phytochemistry

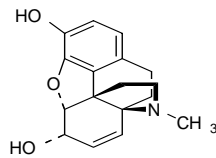
Familiarise yourself with the types of secondary metabolites commonly occurring in plants



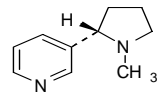
a. Coniine



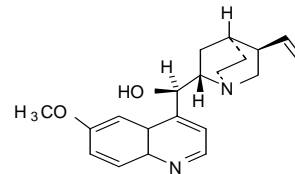
b. Batrachotoxin



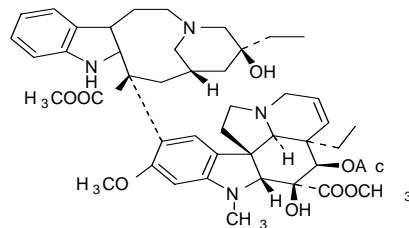
c. Morphine



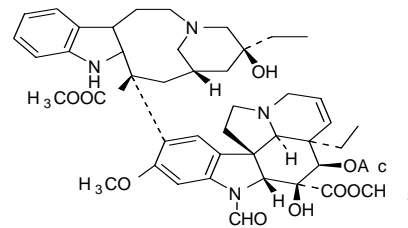
d. Nicotine



e. Quinine



f. Vinblastine



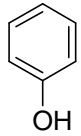
g. Vincristine

Alkaloids

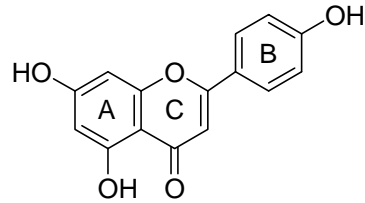
<http://www.biologie.uni-hamburg.de/b-online/e20/20.htm>

<http://books.google.co.uk/books?id=hmYbjbGU-EgC&pg=PA484&lpg=PA484&dq=plant+secondary+metabolite+classification&source=bl&ots=oNhUChMi-r&sig=7hLTjp2x9kTIX9xPusWVLCQC7pg&hl=en#v=onepage&q&f=false>

Phenolic structures

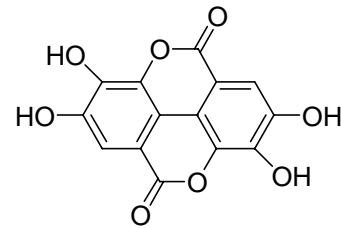
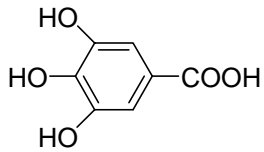


Phenol

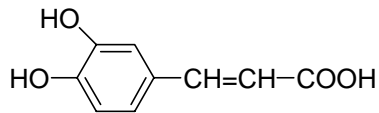


Typical flavonoid

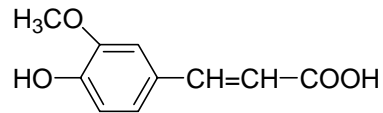
Basic phenolic structures



Phenolic acids, Gallic acid and its dimer ellagic acid

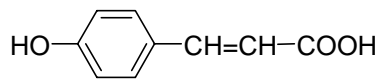


a

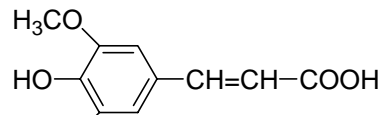


b

Hydroxycinnamic acids caffeic (a), ferulic (b), *p*-coumaric (c), sinapic (d)

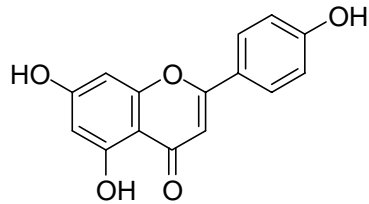


c

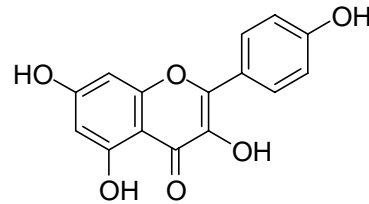


d

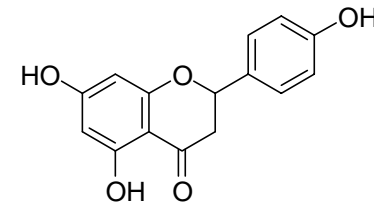
Flavonoids



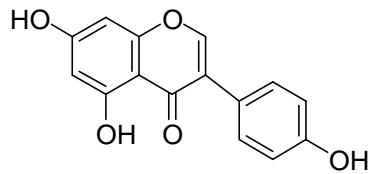
Flavone (e.g. apigenin)



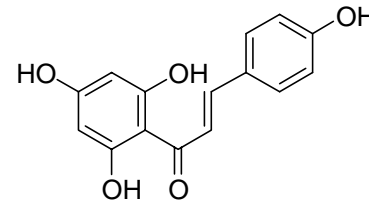
Flavonol (e.g. kaempferol)



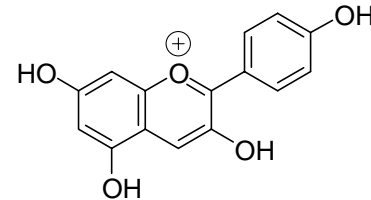
Flavanone (e.g. naringenin)



Isoflavone (e.g. genistein)



Chalcone (e.g. isoliquiritigenin)



Anthocyanidin (e.g. pelargonidin)

Flavonoids are the largest group of naturally occurring phenols, comprising half of all phenolic compounds. They can be found in all plant parts. Chalcone is the common precursor of all the main flavonoid classes and arises from the merging of two main biosynthetic pathways, namely the Shikimate (via phenylalanine to coumarin) and the Acetate-Malonate (via Acetyl CoA to Malonyl CoA). The basic skeleton of most flavonoids contains 15 carbon atoms. The flavonoid carbon skeleton consists of two units. The basic skeleton consists of a C6 phenolic ring known as the A ring and another C6 phenolic ring known as the B ring. These rings are connected by 3 carbons, which often form a 3rd, C ring. However, in chalcones it is a 3-carbon chain.

Flavonoids have characteristic absorption spectra.

Terpenoids

The terpenoids, sometimes called isoprenoids, are a large and diverse class of naturally-occurring organic chemicals similar to terpenes, derived from five-carbon isoprene units assembled and modified in thousands of ways. Most are multicyclic structures that differ from one another not only in functional groups but also in their basic carbon skeletons.

Hemiterpenoids, 1 isoprene unit (5 carbons) (eg isoprene, isovaleric acid)

Monoterpenoids, 2 isoprene units (10C) (eg limonene, pinene)

Sesquiterpenoids, 3 isoprene units (15C) (eg artemisinin, bisabolol)

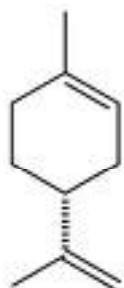
Diterpenoids, 4 isoprene units (20C) (e.g. ginkgolides, taxol, phytol, retinol, forskolin)

Sesterterpenoids, 5 isoprene units (25C)

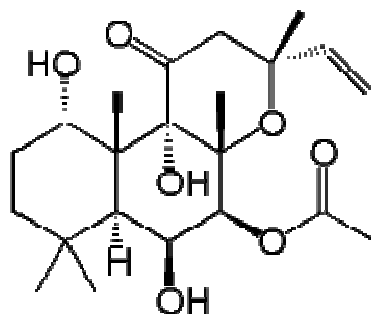
Triterpenoids, 6 isoprene units (30C) (eg lanosterol, squalene)

Tetraterpenoids, 8 isoprene units (40C) (e.g. carotenoids, lycopene)

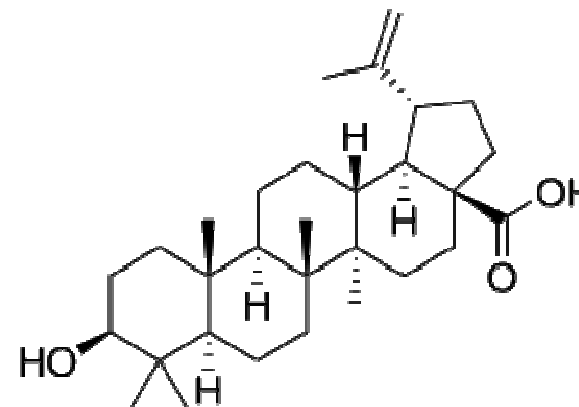
Polyterpenoid with a larger number of isoprene units



Limonene (a monoterpene)



Forskolin (a diterpene)



Betulinic acid (a triterpene)

<http://www.cyberlipid.org/simple/simp00044.htm#2>

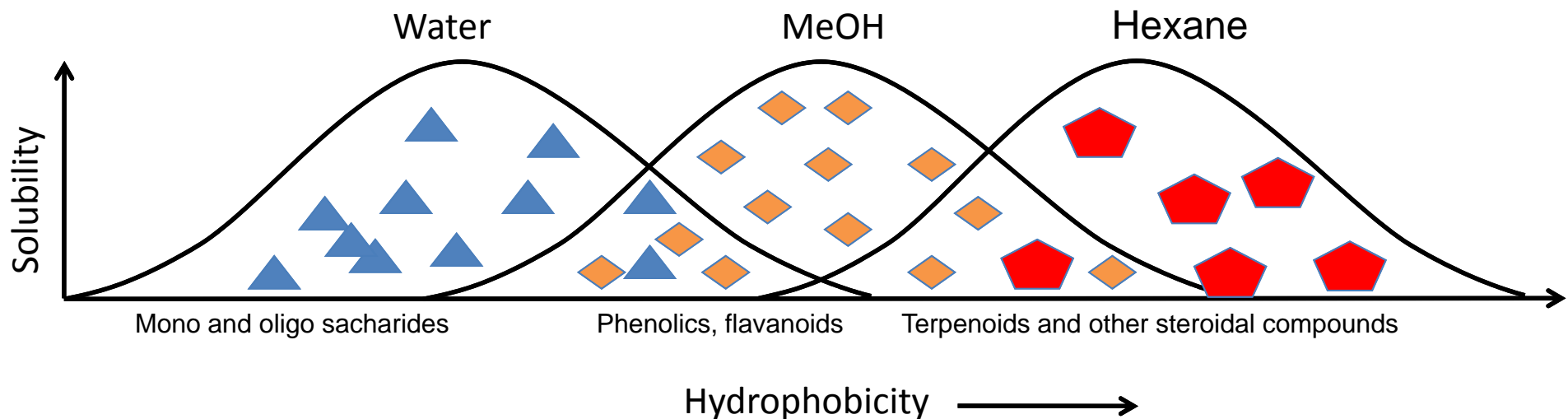
<http://www.chem.qmul.ac.uk/iupac/class/terp.html>

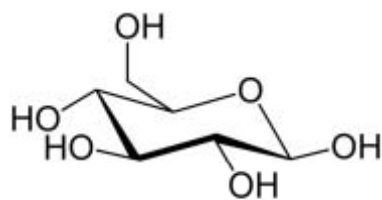
Extracting plant material



Different plant secondary metabolites extract into different solvents depending principally on their ability to form intermolecular bonds with the solvent. Thus polar compounds such as sugars have numerous hydroxyl groups and readily extract into water whilst compounds such as terpenoids with saturated structures lacking polar substitutions such as hydroxyl groups extract more readily into organic solvents such as chloroform. Phenolics such as flavonoids fall between the two and typically extract into aqueous methanol. Extraction like any form of separation is not absolute and there is considerable overlap in the compounds each solvent will extract from a plant. Thus water and methanol extracts of a plant will contain many of the same compounds just at different concentrations. Solvents can be placed in order of their hydrophobicity to form an elutrophic series. An elutrophic series of solvents commonly used in extracting plant material might look as follows:

Water, Methanol, Ethanol, Acetone, Acetonitrile, Ethylacetate, Chloroform, Hexane





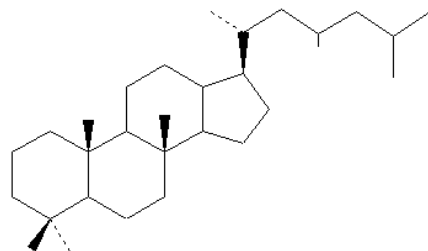
Q. Which layer would these two molecules partition into, (water on top or chloroform below)



Water (Aqueous)

Chloroform (organic)

A. Glucose with its numerous hydroxyl groups would partition into the water layer. The steroidal compound with its aliphatic chain and lack of hydroxyl groups is considerably more hydrophobic and would partition into the organic chloroform layer.



Principles of chromatography

Learn these separation modes of chromatography and in each case research the basis of the interaction between the stationary phase and the analyte and how the mobile phase overcomes the interaction.

Normal phase adsorption

Reverse phase hydrophobic interaction

Size exclusion (gel filtration)

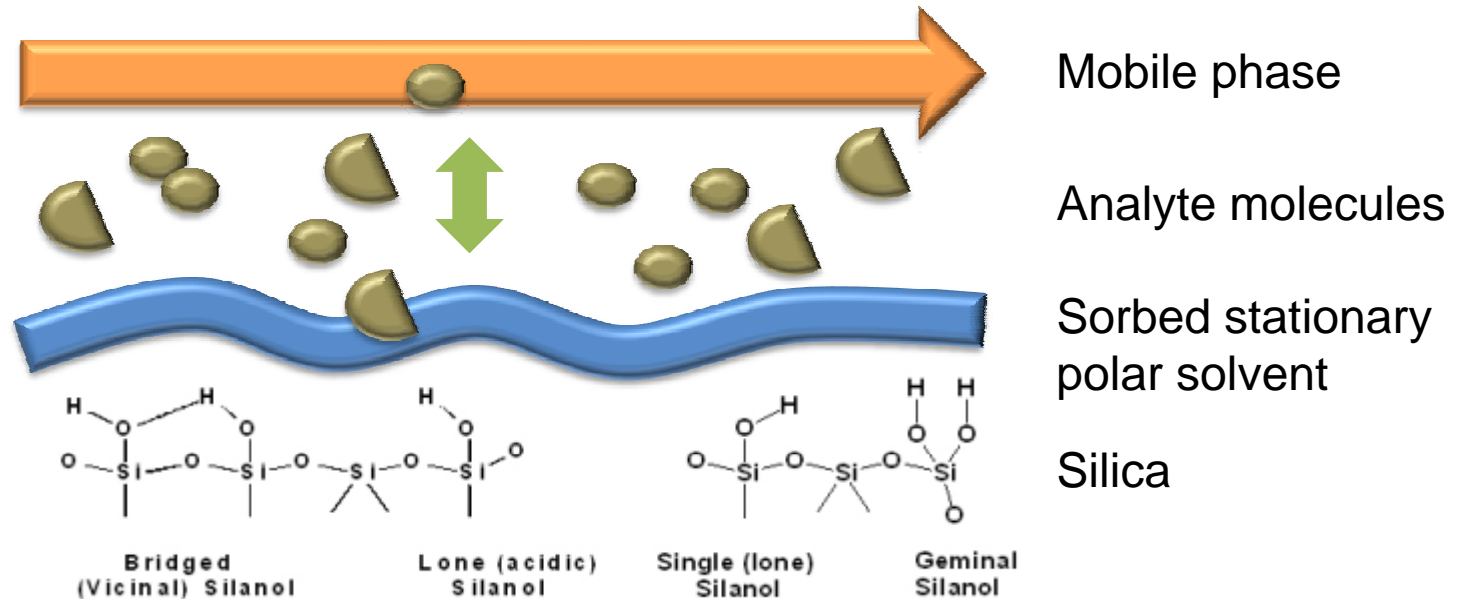
Understand the concepts of stationary phase, mobile phase, solvent gradient etc

<http://en.wikipedia.org/wiki/Chromatography> (read as far as chromatography terms)

<http://www.youtube.com/watch?v=7nFKsmsyugg> (visual demonstration of separation)

<http://www.youtube.com/watch?v=AQ2qpiTGlzI> (watch as far as 1:30)

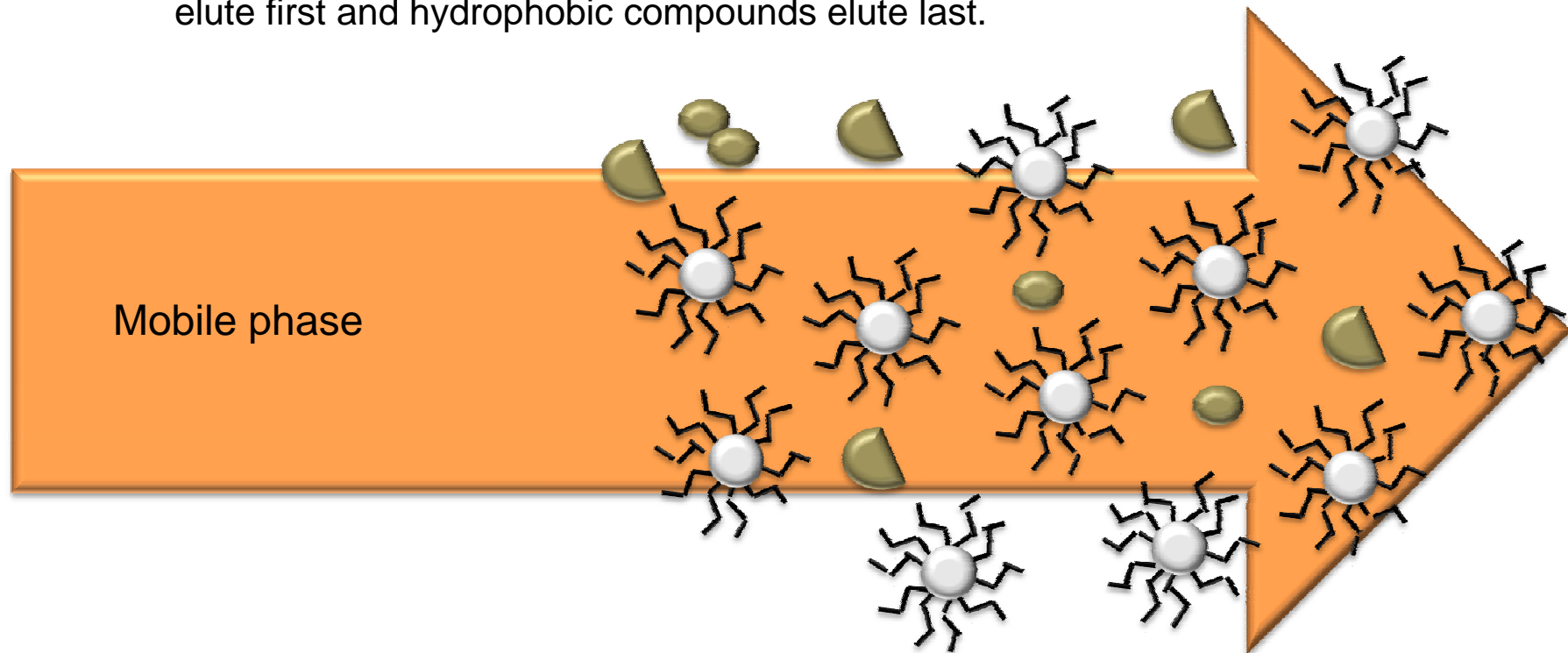
Normal phase on silica



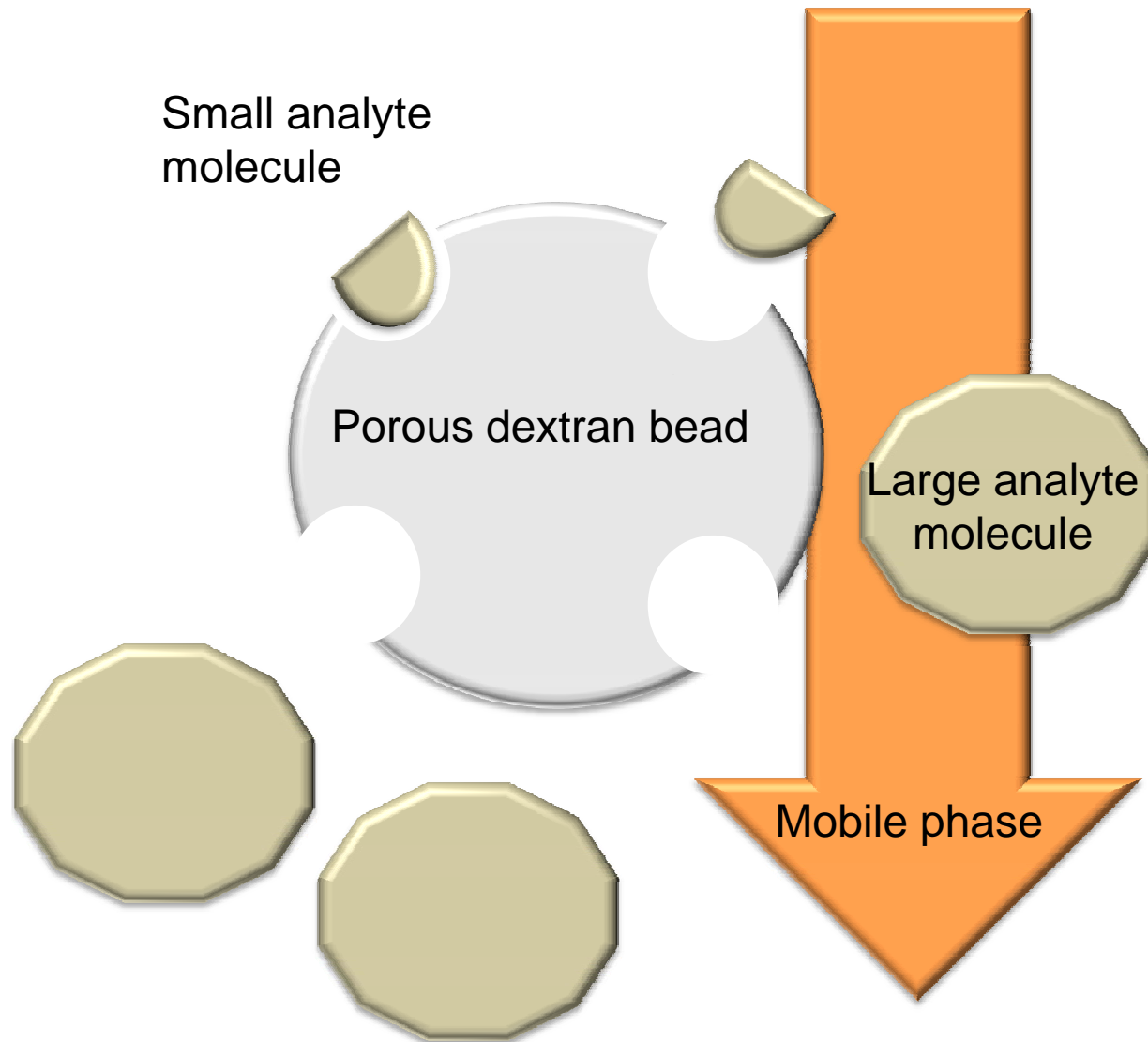
A polar solvent such as water is sorbed onto silica by hydrogen bonding between the silica silanol groups and water. The mobile phase consists of a more hydrophobic solvent. The analytes may then partition into the sorbed water (in effect the stationary phase) or the organic mobile phase. As the mobile phase composition is altered to become more polar the eluting power of the mobile phase increases and analytes are eluted. Thus hydrophobic compounds elute first and hydrophilic compounds last.

Reverse phase C-18 (this is mostly is what we use.

In reverse phase separation, carbon chains (black) typically C4, C8 or C18 are bonded to the silica (white circles) to create a hydrophobic stationary phase. Analyte molecules (grey) may then interact with the stationary phase via hydrophobic interactions. The analyte molecules can either stay sorbed to the stationary phase or as the composition of the mobile phase becomes increasingly hydrophobic (ie increasing organic solvent such as methanol or acetonitrile) migrate with the mobile phase. Thus hydrophilic compounds elute first and hydrophobic compounds elute last.



Size exclusion (gel filtration)



In gel filtration separation occurs by size and shape rather than charge or hydrophobicity. Large molecules pass through the column more rapidly than small molecules which are retained in pores in the surface of the dextran beads that form the stationary phase creating a “reverse sieve” effect. Thus large compounds elute first and small compounds last. In practice gel filtration products applicable to phytochemistry such as **Sephadex LH-20** operate under a mixed reverse-phase size exclusion mechanism

HPLC

Understand the core principles behind HPLC

Seven things you must never do with an HPLC:

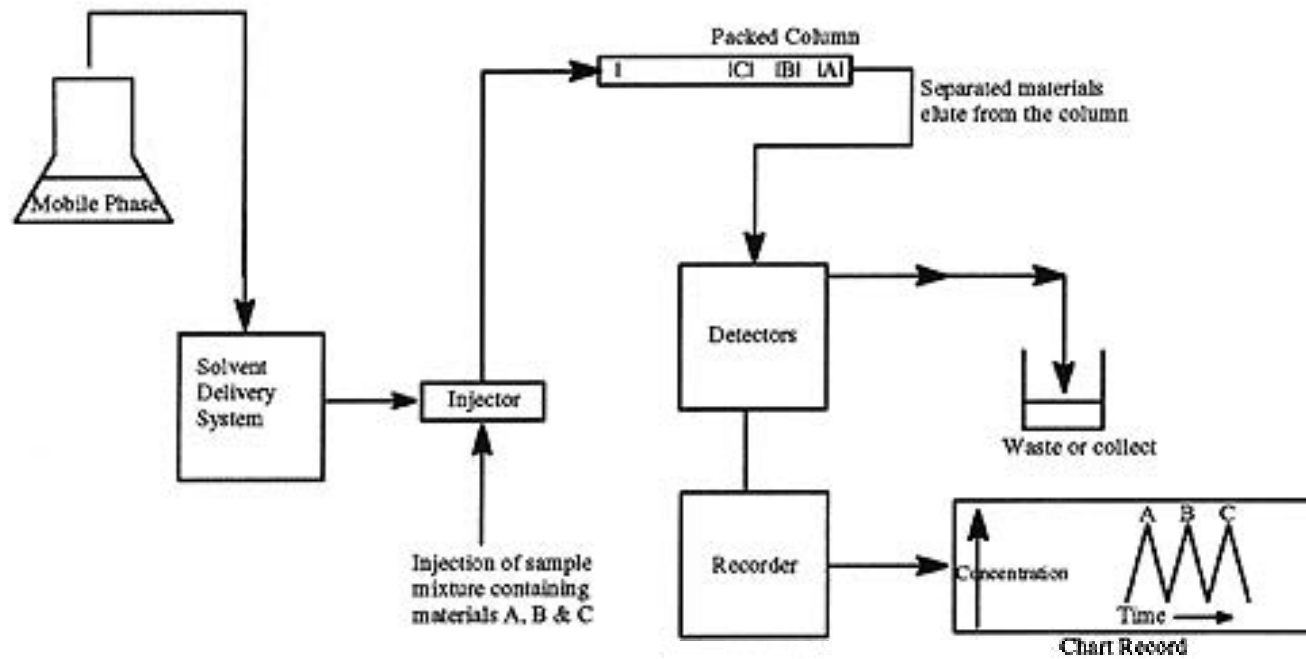
1. Pump 100% water through a C-18 column
2. Inject samples that have not been centrifuged or filtered to remove particles even if they are fractions from another column
3. Change the solvent composition or flow rate too rapidly
4. Pump un-filtered solvents
5. Use a flow rate of more than 1.5ml/minute with an analytical column or 6ml a minute with a semi-prep column
6. Leave running without sufficient solvent
7. Leave machine running overnight without an automatic shutdown



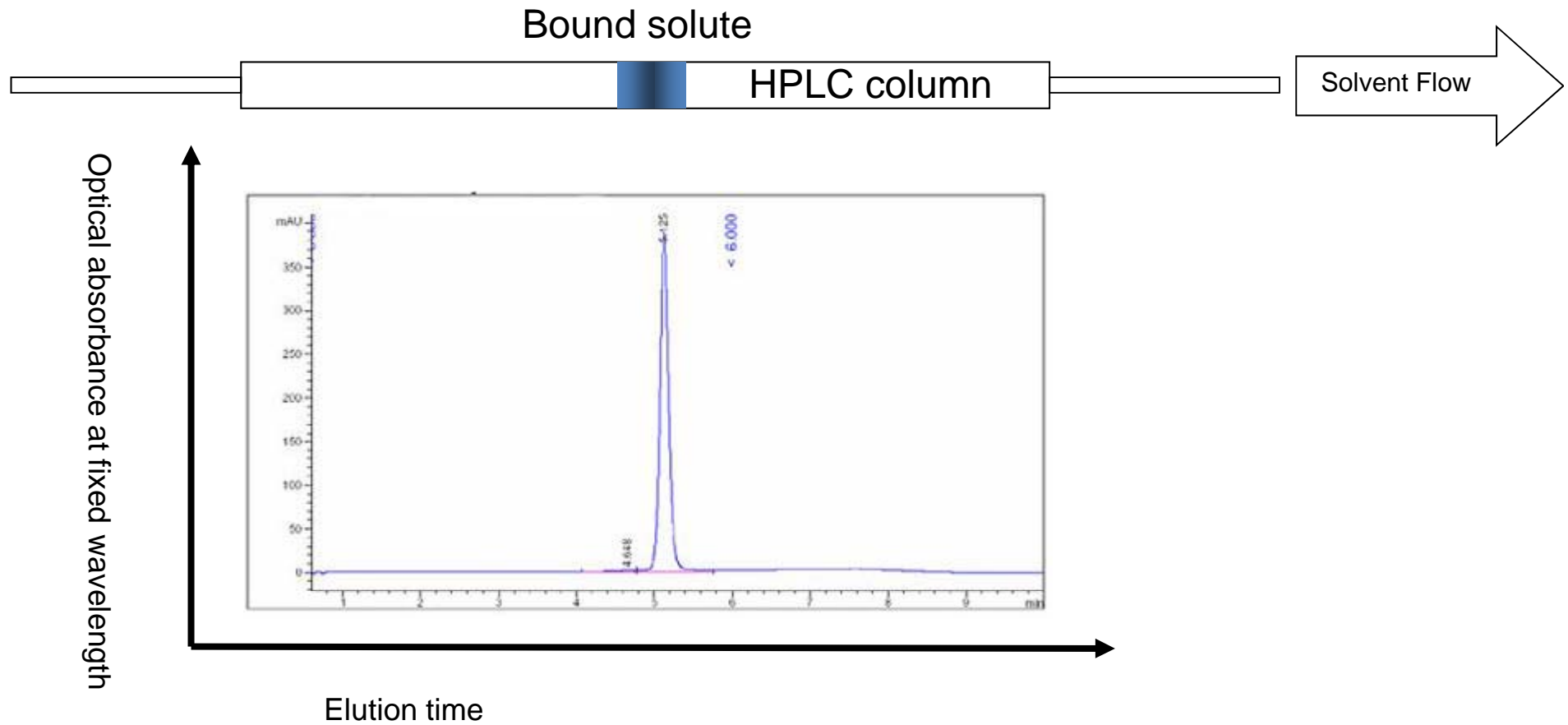
<http://www.youtube.com/watch?v=I-CdTU5X4HA>

HPLC configuration

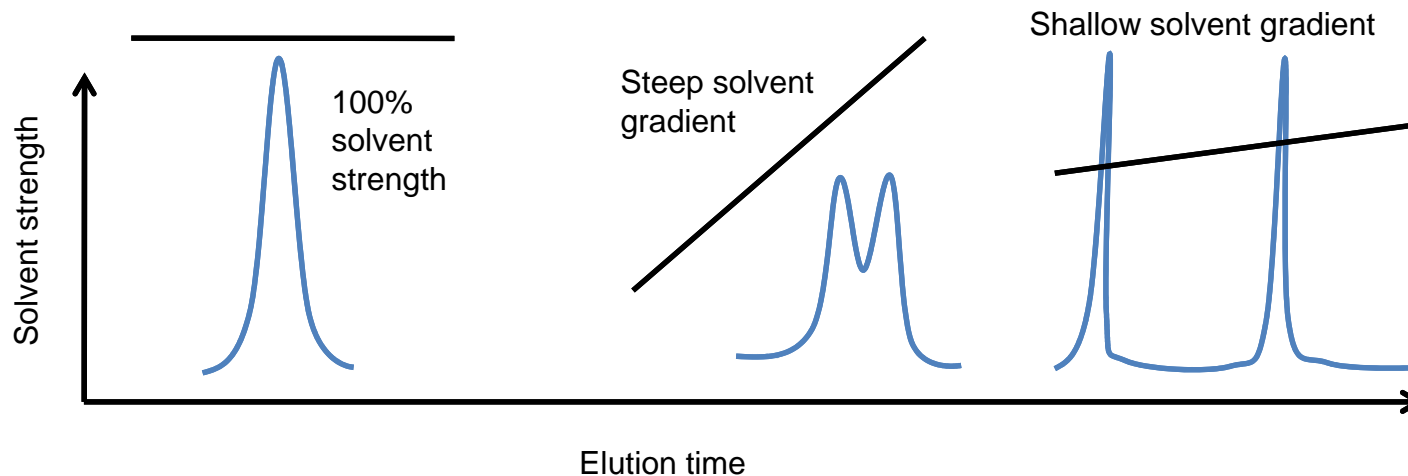
Block diagram showing the components of an HPLC instrument



HPLC peaks arise from the distribution of analyte on the column

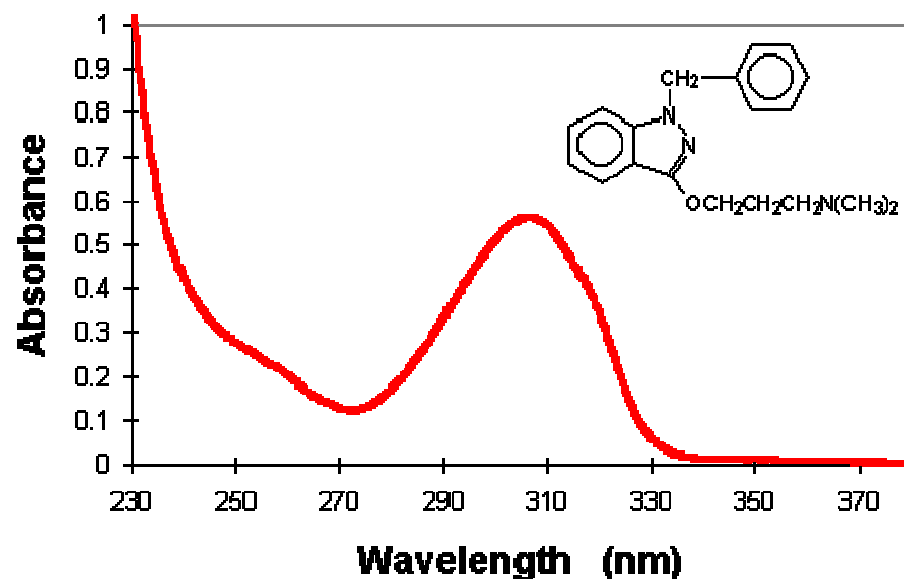


Solvent gradients can be used to improve separation of HPLC peaks



The eluting power of the mobile phase determines how quickly the analytes move down the column. A mobile phase that is 100% organic in a reverse phase system would tend to elute all the analytes together as a single peak (no separation). To increase the “resolution” of the chromatogram you need to use a mobile phase with a solvent composition that is sufficiently strong to elute all the analytes but weak enough to chase them off one by one. Such isocratic elutions (where the solvent composition of the mobile phase stays the same) are not always possible. Solvent gradients (low to high organic in a reverse phase system) are an ideal way of ensuring that analytes separate in the column to produce resolved HPLC peaks.

HPLC photodiode array detectors allow peaks to be detected at many wavelengths simultaneously to produce an absorbance spectrum that may be characteristic of a general compound class



Compounds with conjugated double bonds and therefore delocalised electrons tend to absorb UV light; compounds lacking delocalised electrons such as sugars give no detectable absorbance at all. Some compound classes such as types of flavonoid exhibit characteristic absorbance spectra. Now go back to the compounds in slides 12, 13 and 15 and put them in order of elution on a normal phase HPLC column and a reverse phase HPLC column. State which compounds are likely to have characteristic absorbance spectra or wouldn't absorb at all.

Thin layer chromatography

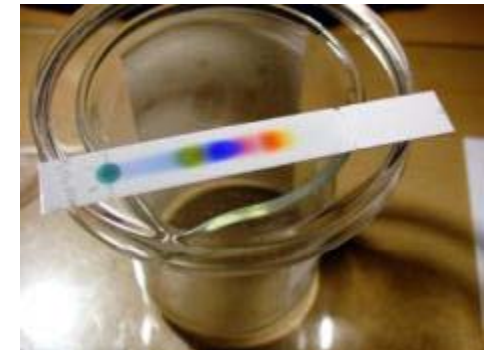
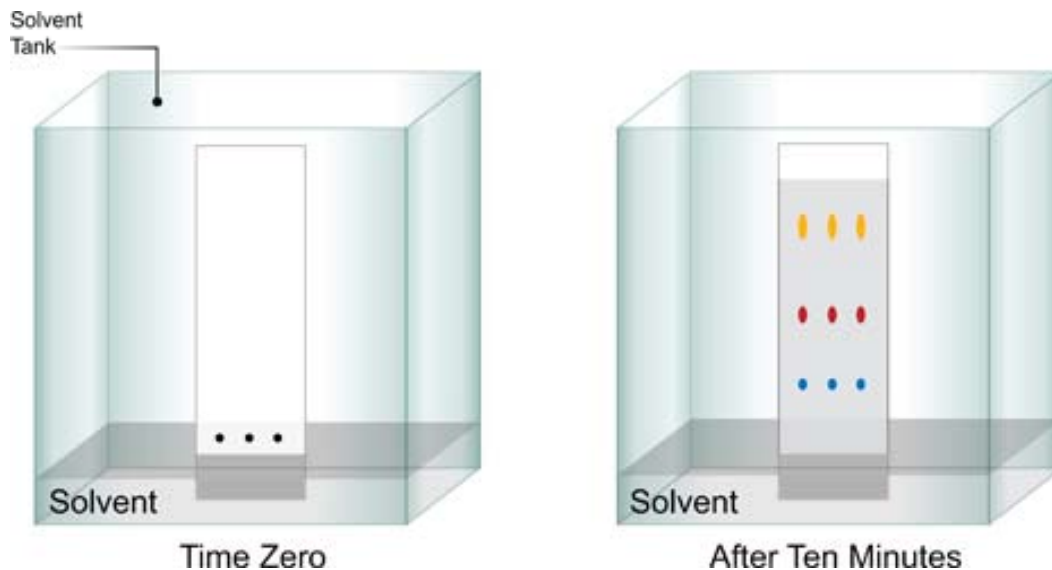
http://en.wikipedia.org/wiki/Thin_layer_chromatography

Allows many samples to be run simultaneously (not possible with HPLC)

Is cheap to set up

Can be harder to resolve compounds than HPLC

Isolating compounds is generally restricted by low amount of compound than can be loaded



Basic general background to Mass Spec (concept only)



Mass spectrometry can be combined “in line” with HPLC to produce LC-MS which gives mass spectral data on each HPLC peak. It is possible to determine the precise molecular mass “accurate mass” of individual compounds in an extract and then identify them on a database such as the combined chemical dictionary.

http://www.youtube.com/watch?v=J-wao000_qM&feature=related

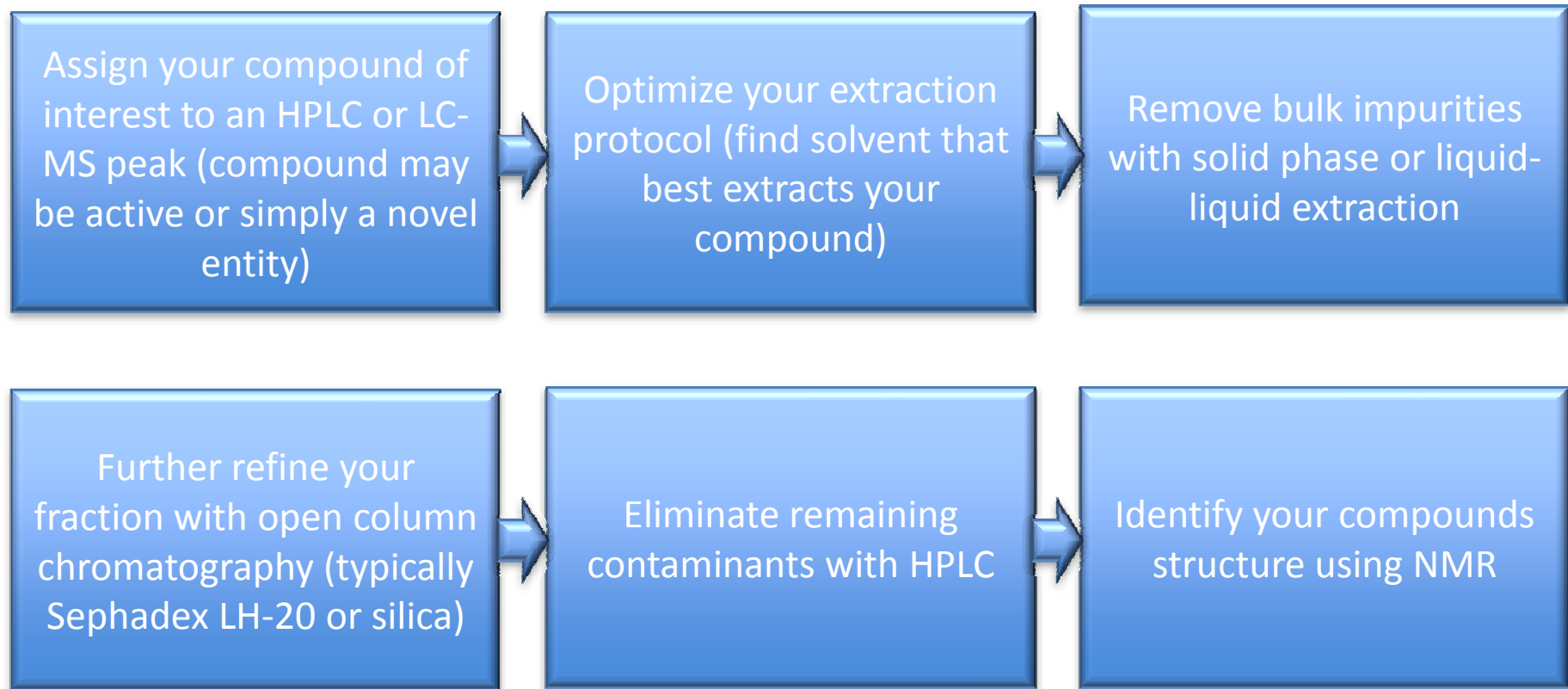
Basic general background to NMR (concept only)



If you have a novel compound or a compound that yields ambiguous mass spectra, NMR will be necessary to fully characterise the compound. In practice the combination of LC-MS, UV spectra and NMR are used to fully elucidate a compounds structure.

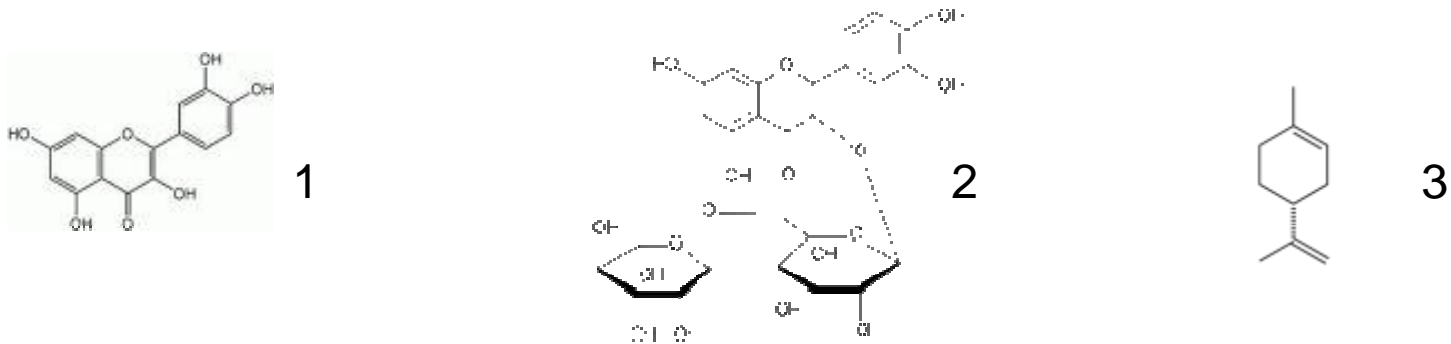
<http://www.youtube.com/watch?v=uNM801B9Y84>

A generic and simplified isolation protocol



Questions

1. When weighing out a compound or reagent what must one avoid doing to avoid contamination
2. When working with organic solvents what precautions must be taken
3. When using Gilson pipettes what must you avoid doing that could un-calibrate or damage them
4. If you were pipetting 180ul and 500ul what pipettes would you use and what colour would the tips be
5. How would you produce a 1M glucose solution, add 1L water to 1M glucose or take up 1M glucose to a final volume of 1L. State your reason.
6. You have 2mg rutin what is the most practical way to prepare a 0.5ml of a 3mg/ml solution in ethanol (how would you weigh out the rutin powder)
7. You have 2.4mg of a pure compound of molecular weight 320, how would you produce a 1mM stock solution and then make 400ul of a 27uM solution in one step
8. You have a 50% methanol extract, what concentration techniques might you use to remove the organic portion of the extract. How would you then remove the remaining water
9. You extract dried leaf material with first water then methanol then acetone and finally hexane. Which extracts would you expect to share the most compounds with one another
10. What flow rates must not be exceeded on analytical and semi-preparative HPLC columns
11. You have 1L of a precious plant extract in a conical flask, what is the most efficient way to determine its concentration in mg/ml solute/solvent



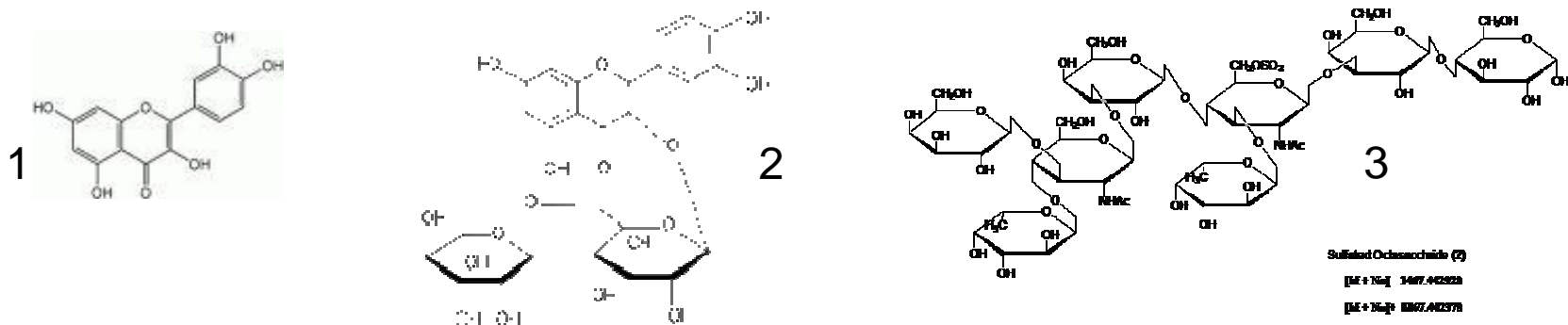
You have a semi-pure fraction containing the compounds shown above

1. Which of these compounds would elute first from a Sephadex gel filtration column
2. Which compound would migrate furthest on a silica TLC plate
3. Which compound would elute first when using reverse phase HPLC
4. You partition the fraction between water and hexane and run the aqueous phase on HPLC using a C-18 column to reveal peaks with retention times 8 minutes and 23 minutes. What compound class does the peak at 23 minutes belong to and does it have a characteristic absorption spectrum.

Congratulations you are now ready
to start work in the lab

Re-sit questions

1. What must you do after weighing out a compound or plant material
2. When making a new solution or extract what should you remember to do to aid identification
3. Some lab consumables are expensive and should be used sparingly give an example
4. If you were making 1ml serial dilutions (1/10 dilutions with a total volume of 1ml) what pipettes would you use (what would they be labelled on the button) and what colour would the tips be.
5. You have a 1mM stock solution of rutin, how would you make 400ul of a 237uM solution in one step (show your working)
6. You have 1L of a non-volatile compound dissolved in a 1:1:1 methanol/acetone/water solution, what is the quickest way to remove the organic portion, suggest an overnight method that will remove the aqueous portion and yield a powdery solid
7. You extract an anti-malarial tree bark with water and find it to be weakly active whereas hexane gives no activity but acetone improves the activity, suggest a solvent to try next.
8. The most active bark extract is run on the LC-MS to reveal a single UV peak whose corresponding mass spectrum suggests a molecular weight which when searched on the combined chemical dictionary returns three matches; a sugar like compound, a terpenoid with numerous aliphatic side chains and a flavonoid. Which of these is the most plausible structure for the active principle in the bark extract, state your reasoning.
9. What concentration of water should never be pumped through a C-18 HPLC column



- Which of these compounds would migrate the least distance on TLC where the stationary phase is C-18 silica
- You run a mixture of all three compounds on an HPLC detecting at a wavelength of 300nm and elute all three compounds but only detect two HPLC peaks, which compounds are they, why don't you see the third peak
- You run the mixture on reverse phase HPLC eluting with 100% methanol but only see 1 peak, you change the mobile phase to 50% methanol and see two peaks, why?
- Which of these compounds would elute first on HPLC where the column is normal phase silica
- Which of these compounds would most readily partition from water into chloroform
- You have a fraction containing the above compounds which you run on a gel filtration column operating under solely size exclusion separation mode. You collect the last fraction and run it on a normal phase TLC plate to reveal two spots. What compound does the spot that has migrated the least correspond to, what compound class does it belong to and does it have a characteristic UV absorbance spectrum.