Yellow Dye Extraction from *Tagetes erecta* (Marigold) Petals.

Materials:

Marigold flowers, dry
Marigold flowers, fresh
Lutein supplement, "Nature's Bounty"
Lutein supplement, "Nature's Basket" (FloraGLO™)
Beta-carotene supplement, "Nature's Basket"
Ethanol
n-Hexane
Ethyl acetate
Acetone
Methanol
KOH
Thin layer chromatography (TLC) plates
TLC tanks
100 ml Erlenmeyer flasks
20, 50, 100 ml measuring cylinders
10 ml pipettes and pipette aid
50 ml Falcon tubes
Funnels and filter paper
Eppendorf tubes and racks
Aluminum foil
Stir plates or flask shaker
Lab centrifuge with swing-out rotor for falcon tubes (optional)
Lab freezer -20C
Chemical Fume hood
Disposable lab gloves
Spectrophotometer
Lab balance
Weight boats
Razor blades or sharp knives
Distilled water
Ice buckets, ice
1ml pipette and tips
capillary tubes
hair dryer
pencils
Saran wrap
Safety goggles
Digital camera

Experiments:

Design your experiment: Which question do you want to ask? Which samples will you have to compare to answer it? What are your experimental controls? Discuss and decide.

1. Extraction of marigold petals with organic solvents:

1. Remove petals from flower heads
2. Weight out 1g dried or 10 g fresh petals, chop petals with knife or blade.
3. Transfer petals to Erlenmeyer flask, label flask with your name and the content
4. Add 50 ml Ethanol
5. Close and wrap with aluminum foil, write your name on foil
6. Transfer to flask shaker
7. Shake ON at R.T.
8. Filter fask content into 50 ml Falcon tube to remove petals. Observe color of extracted petals and of the extract.
9. Remove 2x1 ml of extract into eppendorf tubes, label.
10. Freeze 50 ml sample at -20C, keep one 1 ml sample wrapped in foil on ice if you continue with TLC, or wrap both and freeze if you interrupt here.

2. Extraction of supplement samples:

1) Wear gloves and something to protect your clothes from stains.
2) Label one 50 ml Falcon tube and one Eppendorf tube per sample.
3) Take a single capsule and place on a piece of saran wrap.
4) Carefully cut it open with knife or blade.
5) Use 1 ml pipette to remove as much liquid as possible into 50 ml Falcon tube.
6) Add 20 ml ethanol to tube, shake.
7) Remove 2x1 ml into Eppendorf tube
8) Wrap tubes in aluminum foil, see step 10. above. These will be your standards for the experiments below.

3. TLC analysis of organic marigold petal extracts

- Read: "Basic principles of TLC", class handout.

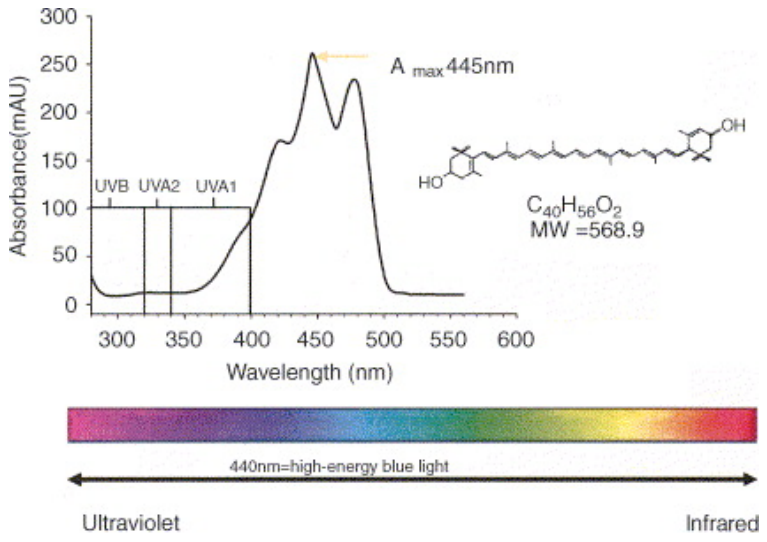
1. Thaw 1ml sample or standard on ice if it was frozen.
2. Prepare TLC tank: **NOTE:** all work with TLC tank and solvents must be performed under the chemical fume hood. Wear lab safety gloves and goggles
    a. Make the solvent: n-Hexane:Ethyl acetate:acetone:methanol 27:4:2:2 (make 100 ml)
    b. Equilibrate the tank: pour solvent ca. 1 cm high into tank, close lid
    c. Prepare TLC plate: Take one plate (wearing gloves) from package and lay down on a clean piece of saran wrap. With pencil, draw a horizontal line 2 cm from bottom of plate. Label the plate in one upper corner. Decide on number of samples and standards to run and label with pencil below the line (leave about 2-3 cm between samples). Set up hair dryer. With capillaries or pipette tips, apply small amount of liquid above each samle number ON the line. Dry after each application. Keep the spot as small as possible. Depending on the concentration of your sample (how deep is your color?) you might have to apply up to 20 times. (work in a place that does not get too much bright light, turn off room lights if possible, work at good speed, your compounds are degraded by light)

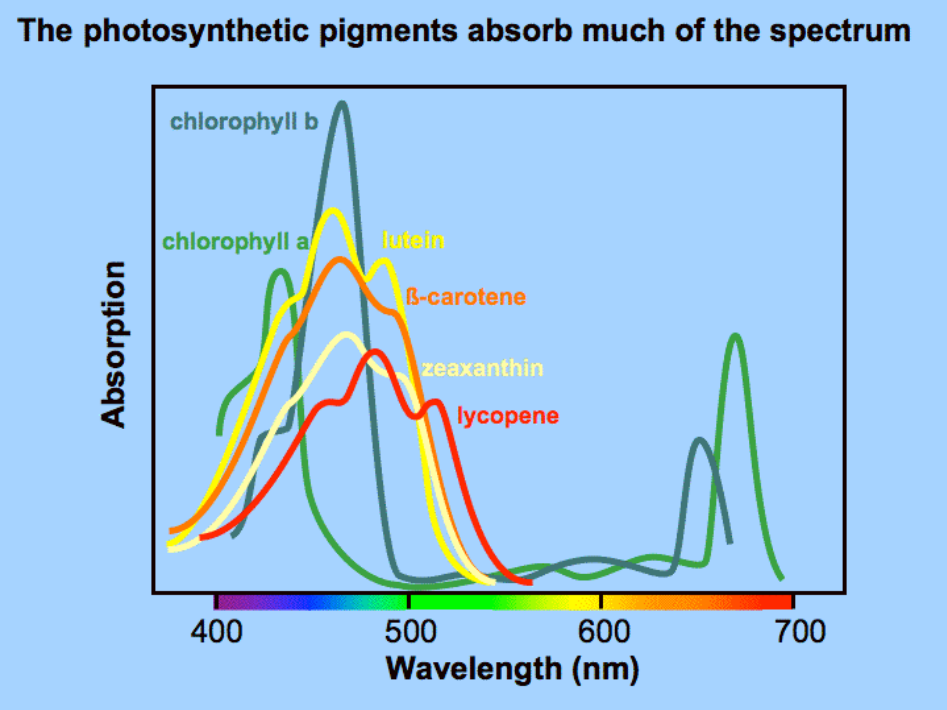
d. Place TLC plate into tank, being CAREFUL to not spill liquid on plate. Spots must be ABOVE the solvent. Watch briefly how solvent migrates up plate and make sure it does so in a staight line. Wrap tank with foil and wait.
e. At end of chromatography (solvent about 1-2 cm from upper rim, ca. 30 min), remove plate from tank, lay down on saran wrap, and with pencil mark the upper egde of the solvent, and circle all yellow spots. Let plate dry competely under the hood.
f. Wrap plate with Saran wrap, remove from hood and photograph to document results. The yellow spots will slowly fade once your plate is exposed to light.
g. Calculate the Rf values of all compounds.
h. Interpret your results. Expected Rf values: Beta-Carotene, 0.96; Lutein, 0.247; Violaxanthin, 0.16; Neoxanthin, 0.087.

4. Spectrophotometry

Read: "Principles of Spectrophotometry" (class handout)

a) Turn on instrument at least 15 min before use
b) Prepare one blank sample (the solvent) and the samples to be measured (your color extracts and standards, and 1:10 dilutions in solvent)
c) Run a spectrum for each sample from 300 nm to 800 nm (use of instrument will be demonstrated). Compare your results to the spectra below.





1. Do the standards match the expectation? If not, what might that mean?
2. What do you think your extracts contain?

5. Saponification of Lutein extracts:

Read: Kimura et al. (1990) Assessment of the Saponification Step in the Quanitative Determination of Carotenoids and Provitamins A. Food Chemistry 35, 187-195.

Lutein in the extract might be in form of fatty acid esters, which would affect its mobility in TLC (did your sample have a spot with the same Rf as the Lutein standard?). Saponification will cleave the chemical bonds between Lutein and fatty acids the same way the soap-making process works (read up on soap making, if you like).
NOTE: 60% KOH is a very strong lye. Take caution to not spill any on your hands, gloves, or clothes.

1. Make a 60% solution of KOH in H2O.
2. Use 25 ml of your ethanol extract and 1-2 ml each of your standards.
3. Add 1 ml of KOH solution per 10 ml of extract.
4. Leave solutions in dark at room temperature for 12-16 hours.
5. Repeat TLC to compare extracts and standards before and after saponification (remember during all steps that some of your samples contain lye).
6. Interpret your results.

6. Crystallization of Lutein.

Read: Patent #5382714. Process for isolation, purification, and recrystallization of Lutein from saponified marigold oleoresin and uses thereof.

1. Calculate the ethanol:water ratio in your saponified extracts.
2. Add distilled water to obtain the ratio of water:ethanol=2.3:1
3. Incubate you sample on ice or in the fridge (4C).
4. Carotenoids are minimally water soluble. At this ratio, you should see a precipitate or orange crystals form in the cold. (The precipitate will also contain KOH from the saponification step.)
5. Filtrate precipitate and wash with ice cold water on filter.
6. Dry crystals, and observe by microscopy (will be demonstrated). The image below shows a published micrograph of marigold Lutein crystals (Kemin Foods L.C., Des Moines, Iowa). Compare your result and discuss. Have you just made your own FloraGLO™? Why would you/ would you not want to use it as a health supplement?