METHODS OF POLLUTION CONTROL AND WASTE MANAGEMENT

Experiment no. 33

Pollution of the aquatic environment with steroid hormones

Occurrence, removal and determination of androgens

MANUAL

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Requirements:

1. Definition of an endocrine disrupting chemicals (EDCs)
2. What effects the EDCs can cause in animals and humans?
3. How the EDCs can interfere with the endocrine system?
4. Processes that can remove the most of EDC in waste water treatment plants
5. Why the relatively higher efficiency of EDC removal using NF compared to UF is observed?
6. Names of group of steroid hormones; one example of a compound belonging to each group
7. The presence of estrogenic compounds in water, their deleterious effect and at least 3 examples of their removal.
8. Short characteristic of other steroid hormones, including glucocorticoids, androgens, and progestogens
9. Where most of glucocorticoids, androgens, progestogens and estrogens was eliminated in a municipal sewage treatment plant (STP)?
10. Nine androgens, nine progestogens, and five estrogens were analyzed in influent and final effluent wastewaters in seven wastewater treatment plants (WWTPs) of Beijing, China in 2006.
    a. which group of compounds was present in the highest and lowest concentrations?
    b. what was a removal efficiency of androgens, progestogens and estrogens?
11. What are the methods for the measurements of steroids hormones at a trace level in wastewater samples?
12. What does the chromatography consist in?
13. The Lambert-Beer Law

Ultraviolet and visible light range (UV/VIS) is widely applied in research, production and quality control for the classification and study of substances. Steroid hormones containing similar functional groups in their structure will have the same or very similar λ_{max}. Therefore, to calculate the concentration with the Lambert-Beer Law they have to be separated first. For this purpose we will use reverse-phase liquid chromatography.

Column chromatography is one of the most useful techniques for purifying compounds. This technique utilizes a stationary phase, which is packed in a column, and a mobile phase that passes through the column. A type of a liquid chromatography performed using nonpolar solid support stationary phase (unmodified silica or alumina resins) is called a normal-phase chromatography. Reversed-phase chromatography is a technique using alkyl chains covalently bonded to the stationary phase particles (for example C-18 alkyl) in order to create a hydrophobic stationary phase, which has a stronger affinity for hydrophobic or less polar compounds. Reversed-phase chromatography employs a polar (aqueous) mobile phase. As a result, hydrophobic molecules in the polar mobile phase tend to adsorb to the hydrophobic stationary phase, and hydrophilic molecules in the mobile phase will pass through the column and are eluted first. Hydrophobic molecules can be eluted from the column by decreasing the polarity of the mobile phase using an organic (non-polar) solvent, for example methanol, which reduces hydrophobic interactions. The more hydrophobic the molecule, the more strongly it will bind to the stationary phase, and

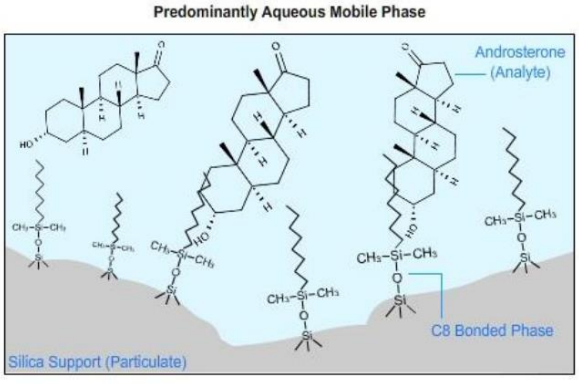
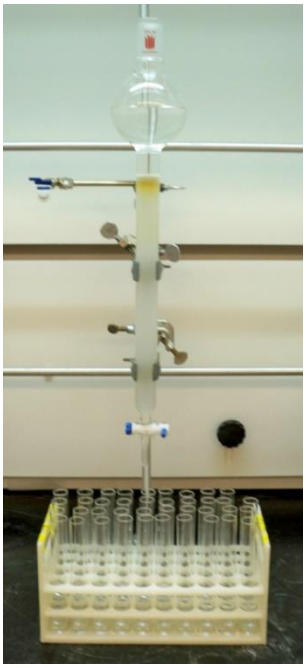


Figure 1: Schematic representation of reversed phase HPLC.

the higher the concentration of organic solvent that will be required to elute the molecule. C-18 alkyl chain phase is used to trace organics in environmental water samples.

How to Perform Column Chromatography

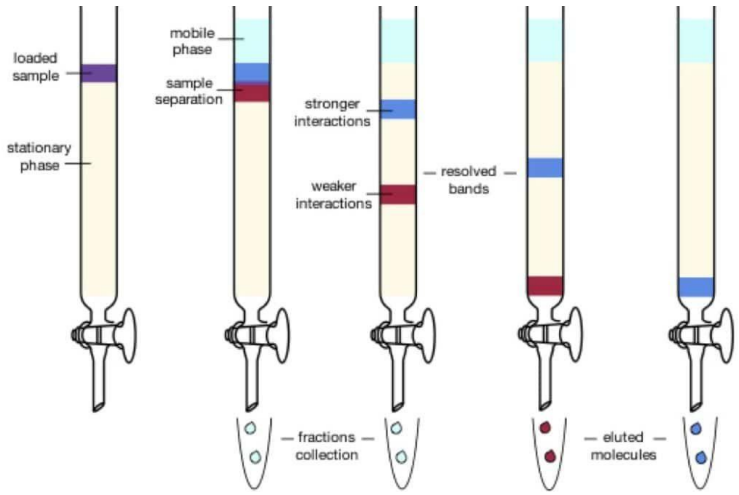
1. Using a ring stand and an appropriate clamp, position the Seppack C-18 (plastic column already filled with stationary phase) so that the tip of the column is 1 cm above the top edge of the Erlenmeyer flask.



2. Fill the column using 10 ml of water.
3. Pour the prepared water sample into the column so that the liquid level reaches 1 cm below its edge. Using the pump, push the liquid through the column so that its level reaches 1mm above the stationary phase. Attention!!! Remember, never allow the solvent level to fall below the top layer of stationary phase.
4. Repeat until all the solution has been used up. Rinse the flask with water and pour the resulting liquid back into the column.
5. Start elution using gradient of mobile phase. Begin collecting the mobile phase as it drains from the column into test tubes. Test tubes should be placed in a test tube rack in a sequential manner.
6. Use an eluent that is a mixture of water (W) and methanol (MeOH) with the following volume ratios:

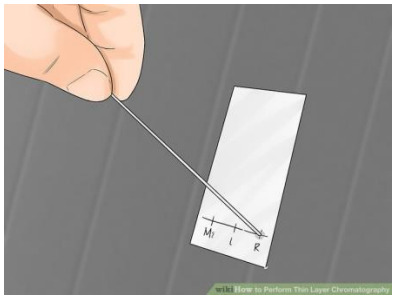
10 ml of W:MeOH 7:3
10 ml of W:MeOH 6:4
10 ml of W:MeOH 5:5
20 ml of W:MeOH 4:6
30 ml of W:MeOH 3:7 - start collecting half a tube
60 ml of W:MeOH 2:8
20 ml of W:MeOH 1:9

Starting with the low content of the organic component in the eluent we allow the least retained components to be separated. Strongly retained components will sit on the adsorbent surface on the top of the column, or will move very slowly. When we start to increase an amount of organic component in the eluent then strongly retained components will move faster and faster, because of the steady increase of the competition for the adsorption sites.

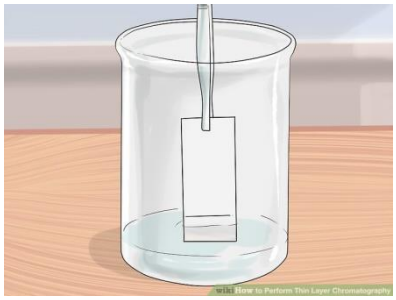


7. Analyze the purity of the eluted solutions using Thin-Layer Chromatography.
8. Prepare your chromatography solvent – 50 ml of mixture W:MeOH 2:8.
9. Pour the solution prepared in this way into the chromatographic chamber
10. Draw a line in pencil about 1 cm from the bottom straight across. Draw evenly spaced vertical dashes on the horizontal line (as many as you have tubes, plus 2 on the contamination patterns). Label these lines.

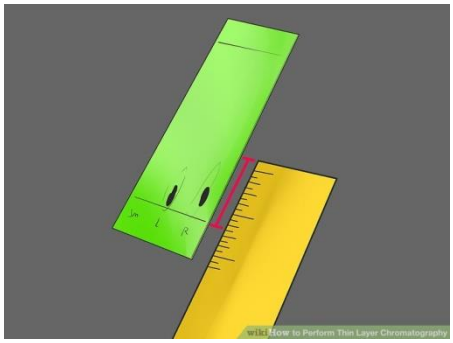
11. Take a small amount of solution. Simply stick the end of the pipette into the liquid, and some of it will come into the pipette.
12. Lightly press the tip of the pipette to the intersection of one of the dashed lines and the horizontal line you made previously on the silica side. This will cause the solution in the pipette to be sucked onto the silica, resulting in a small circle of solvent and compound. Dry it and press the tip of the pipette again, dry and do it one more time.



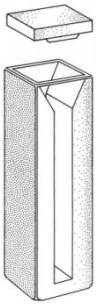
13. Place the plate in the chromatographic chamber, close the lid and wait until the eluent reaches 1 cm below the upper edge of the plate. Then remove the plate using tweezers, lay it on the counter and air dry for 1 minute.



14. Quickly mark in pencil the highest place the solvent reached. This is called the solvent front and is important to take note of for later calculations. Do not touch the silica with your fingers as you could smudge the spots or deposit compounds from your hands onto the plate, making your plate useless.
15. Place the plate under an ultraviolet light. You should notice several spots at different locations, corresponding to different compounds. Use a pencil to outline the spots so you can continue to analyze the plate after you remove it from the UV source.
16. Calculate the retardation factor (Rf). This is simply the ratio of how far the spots have moved to how far the solvent has moved. Use a ruler to measure the distance to the solvent front and to the middle of the spots. Divide the distance the spots moved by the distance the solvent moved. This will give you a number between 0 and 1. By calculating the Rf of known compounds, you can identify them on a TLC plate. Different compounds will almost always have different Rf values, making them easy to distinguish.



17. Pour the fractions containing the same compound into the Erlenmeyer flask. Add magnesium sulfate (desiccant) to cover the bottom. Then pour the solution into the round bottom flask through the funnel with a crimped filter. Rinse the flask and the filter with a small amount of methanol from a wash bottle.
18. Remove the solvent using a rotary evaporator.
19. Prepare the solution by dissolving the resulting compound in the appropriate amount of ethanol.
20. When using a spectrophotometer to determine concentration of a sample solution of unknown concentration by UV/VIS spectroscopy, a calibration line must first be created. This is done by measuring the light absorption of several standard solutions of different, known concentrations at a predefined, fixed wavelength. Once the calibration line is established, the unknown concentration of a sample con be determine by plotting in the above graph.
21. Using the Pasteur pipette, add the resulting solution into the UV/VIS cuvette



22. Insert the cuvette into the spectrophotometer and record the UV/VIS spectrum.
23. The calculation of concentration is governed by the Lambert-Beer Law. To calculate the concentration:

$$C = A / \varepsilon \text{ (epsilon) x } d$$

Where
C =The sample concentration in mol / L or g / mL,
D = Cuvette path length in cm (1 cm)
ε = (epsilon) sample specific constant (describing how much the sample absorbs at a given wavelength).

Testosterone: $\lambda_{max} = 241$; $\varepsilon_{max} = 15{,}800$(EtOH)

Testosterone 17-propionate: $\lambda_{max} = 241$; $\varepsilon_{max} = 16{,}900$(EtOH)

Progesterone: $\lambda_{max} = 241$; $\varepsilon_{max} = 17{,}000$(EtOH)

24. Dispose of solid and liquid byproducts in the appropriate waste containers. Wash all equipment and return it to its proper place.