METHODS OF POLLUTION CONTROL AND WASTE MANAGEMENT

Experiment no. 34

Pollution of the aquatic environment with steroid hormones

Removal and identification of selected steroids

MANUAL

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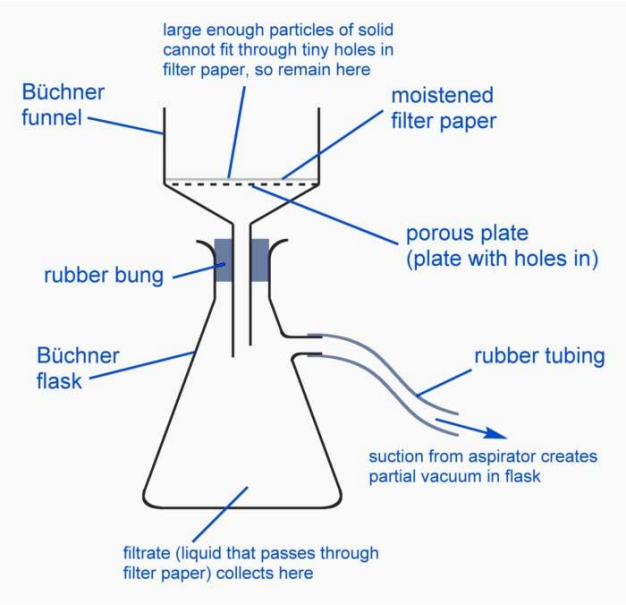
Requirements:

1. Definition of an endocrine disrupting chemicals (EDCs)
2. What effects the EDCs can cause in animals and humans?
3. Names of EDCs classes constituting water pollution; one example of a compound belonging to each class
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. Short characteristic of steroid hormones groups
7. What are the main hormonal contamination from dairy farms
8. Where most of glucocorticoids, androgens, progestogens and estrogens was eliminated in a municipal sewage treatment plant (STP)?
9. 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?
10. What are the methods for the measurements of steroids hormones at a trace level in wastewater samples?
11. What is the derivatization process and when do we use it?
12. Extraction and distribution ratio?

VACUUM FILTRATIONS

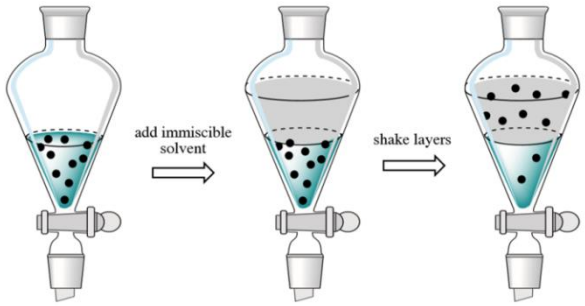
1. Prepare a Büchner funnel fitted with the appropriate size filter paper and rubber bung/stopper; a clamped filter flask (Büchner flask), and a vacuum applied to the side arm of the filter flask.



2. First, make sure you use the appropriate size filter paper. The paper should smaller in diameter than the base of Buchner (but it must cover all the holes) and should sit flat on the bottom of the funnel with no creases or folds.
3. Moisten the filter paper with a little of water to adhere the paper to the funnel
4. Turn on the aspirator tap to its maximum; apply a vacuum to a filter flask with a side arm adaptor
5. Swirl the mixture of the solid and liquid and then pour it quickly into the filtration apparatus
6. Wash the flask with 20 ml of dichloromethane two times
7. Disconnect the tubing before turning off the aspirator tap

EXTRACTION

Extraction in chemistry is a separation process consisting in the separation of a substance from a matrix. Common examples include liquid-liquid extraction, and solid phase extraction. Liquid-liquid extractions in the laboratory usually make use of a separatory funnel, where two immiscible phases are combined to separate a solute from one phase into the other, according to the relative solubility in each of the phases. Typically, this will be to extract organic compounds out of an aqueous phase and into an organic phase.



Distribution ratio

In solvent extraction, a distribution ratio is often quoted as a measure of how well-extracted a species is. The distribution ratio (Kd) is equal to the concentration of a solute in the organic phase (C_{org}) divided by its concentration in the aqueous phase(C_{aq}).

$$K_d = C_{org}/C_{aq}$$

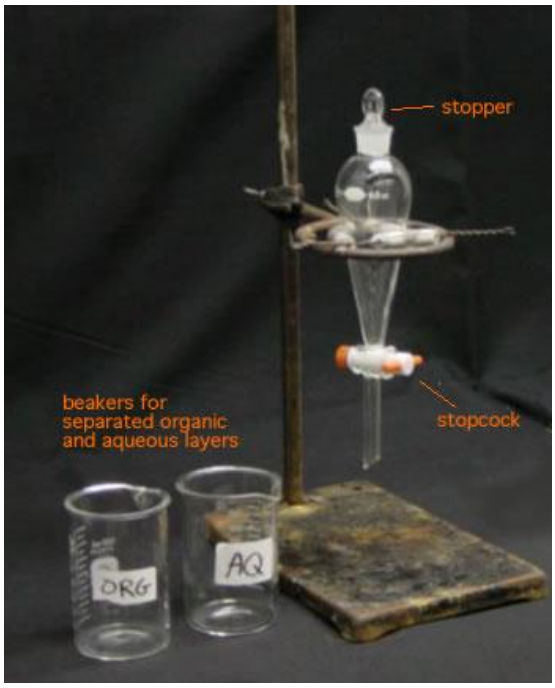


Figure 1. A separatory funnel in a ring clamp attached to a ring stand

PERFORMING AN EXTRACTION

1. Place the separatory funnel in a ring clamp attached to a ring stand or latticework (Figure 1).

2. Before pouring anything into a separatory funnel, be sure that **the stopcock is in the "closed"** position, where the stopcock is horizontal. As a fail-safe, always position an Erlenmeyer flask beneath the separatory funnel before pouring. This can catch liquid in case the stopcock is accidentally left open, or if the stopcock is loose and liquid leaks through unintentionally.

3. Using a funnel, carefully **pour the filtrate** from the filter flask, to be extracted into the separatory funnel. The funnel should not be filled more than half-full. Wash the flask with 20 ml of dichloromethane and add the solution into separatory funnel.

4. **Add 20 ml of dichloromethane (extraction solution)**. The volumes can be measured in a graduated cylinder.

5. **Place the stopper on the funnel**, and hold the funnel such that the fingers of one hand securely cover the stopper, while the other hand grips the bottom of the funnel (Figure 2a).

6. **Gently invert the funnel** (Figure 2b), and vent (**open the stopcock**) away from yourself and others (Figure 2b). You will hear a kind of whoosh when the pressure is released.

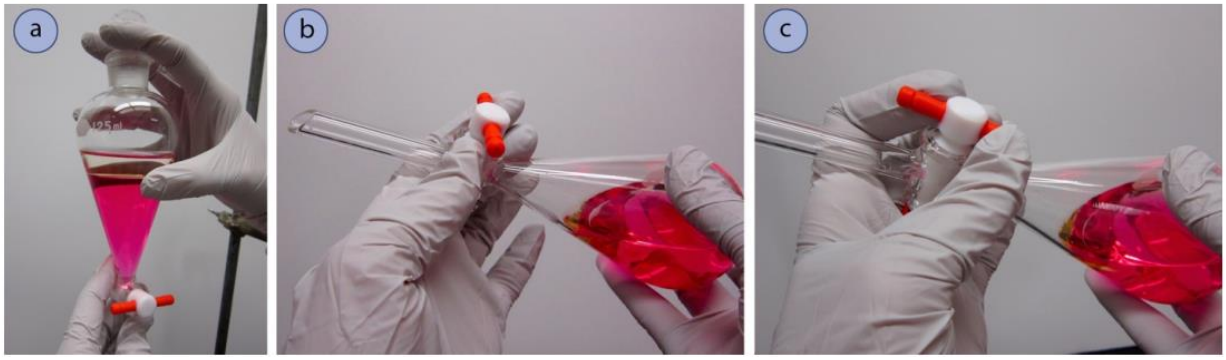


Figure 2. a) Holding the separatory funnel before shaking, b) Inverting the funnel to mix the components, c) Venting to release pressure.

7. **Close the stopcock and shake the funnel gently.** Vent it again. Repeat this step until no more gas escapes. With some solutions (e.g. dichloromethane), care should be taken to not shake too vigorously, as these solutions often form emulsions (where the interface between the solutions doesn't clarify).
8. **Place the separatory funnel upright in the ring clamp** to allow the layers to fully separate. If the interface is clouded remove the stopper.

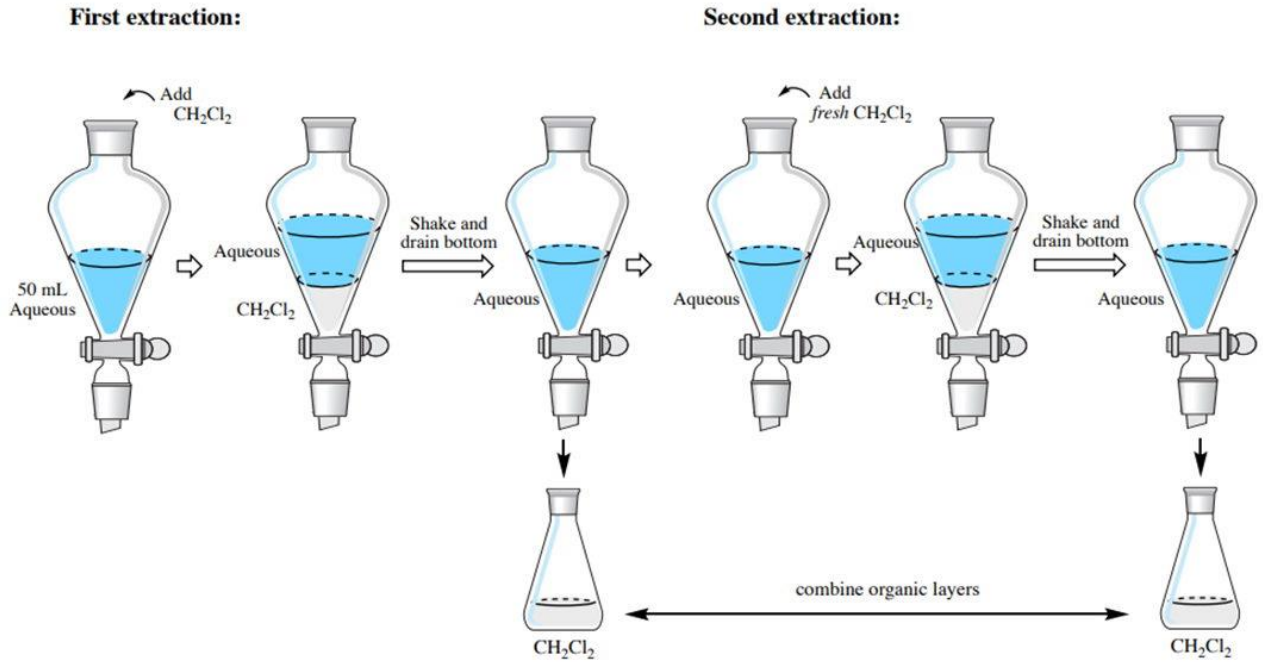


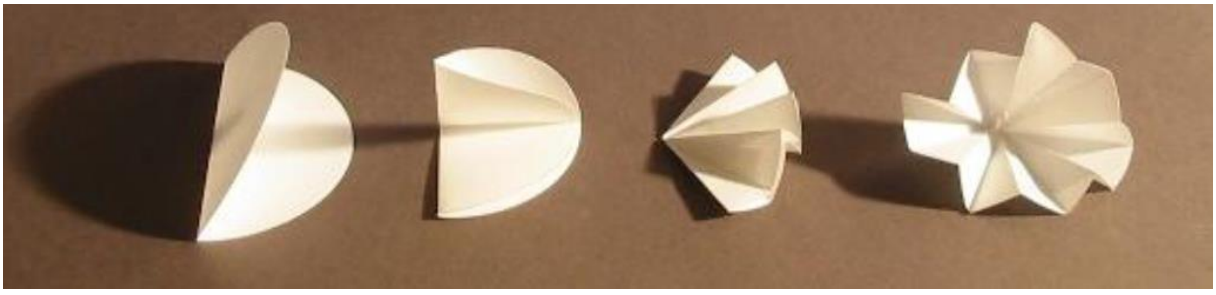
Figure 3. Two extractions when the organic layer is on the bottom.

9. When **the layers do not separate** and you see that tiny droplets of one layer are suspended in the other layer, resulting in no distinct interface between the two layers - an emulsion has formed.

Often an emulsion looks like a bubbly mess near the interface, and can even appear to be an odd-looking third layer.

- For mild emulsions, gently swirl the layers and try to knock down suspended droplets with a glass stirring rod.
- Allow the solution to sit for a period of time.
- If an emulsion occurs with an aqueous solution (top layer) and dichloromethane (bottom layer), add some water from a squirt bottle to dilute the top layer and decrease its density

10. **Drain the majority of the bottom layer** (dichloromethane layer with some amount of steroid hormones) into a clean Erlenmeyer flask, positioning the ring clamp so that the tip of the separatory funnel is nestled in the Erlenmeyer flask to prevent splashing. Stop draining when the interface is within 1cm of the bottom of the stopcock. Gently swirl the funnel to dislodge any droplets clinging to the glass. Further drain the bottom layer, stopping when the interface just enters the stopcock chamber. Label the Erlenmeyer flask (e.g. "bottom layer").
11. **The aqueous layer remains in the funnel** after first extraction.
12. **Add a *fresh* 30mL portion of dichloromethane** to the separatory funnel. Stopper the funnel, invert and shake with venting, then allow the layers to separate. At this step, there should be two layers in the separatory funnel.
13. **The bottom organic layer can be drained** from the separatory funnel into the same flask that was used for the organic layer in the first extraction (that may have been labeled "bottom organic layer").
14. Repeat the extraction by adding another fresh 30mL portion of dichloromethane to the aqueous layer in the separatory funnel. Stopper the funnel, invert and shake gently with venting, then allow the layers to separate.
15. Drain the bottom organic layer into the flask used previously.
16. Then, add a tablespoon of anhydrous magnesium sulfate to the combined organic layers to remove trace water.
17. **The steps to flute the filter paper.** First, fold in half; open and fold in half at 90° to the first fold, subsequently align adjacent folds and make new folds bisecting the previous folds until a fan-like arrangement is obtained. Pleat into a fan by folding each segment in the opposite direction to its neighbors, in accordion-like fashion. When opened out the complete fan-like fluted paper results.



18. Remove drying agent using gravity filtration with a fluted filter paper to the round bottom flask.
19. Remove the solvent with a rotary evaporator.

GAS CHROMATOGRAPHY

The level of steroid hormones in surface water or even in wastewater is so low that is a challenge to measure them. Therefore, there has been a great need to develop a convenient and sensitive method for the measurements of steroids hormones at a trace level. This method should include enrichment and determination of target analytes. ELISA (the enzyme-linked immunosorbent assay) is a sensitive method for the determination of steroid hormones, but is limited by the requirement to develop analyte-specific antibodies. LC (liquid chromatography) or HPLC (high pressure liquid chromatography) has been widely employed for measurements of steroid hormones in the aquatic environment, and it is usually coupled

with MS, UV or fluorescence detection. GC (gas chromatography) has often been used to determine steroid hormones since derivatization was used prior to analysis. Chemical derivatization is used to convert the nonvolatile steroids, due to their high molecular weight and lack of active groups, into forms that are easily chromatographed. Common reagents associated with derivatization involve trimethylsilyl esters, which react to the functional group(s) of the analyte.

1. **Derivatization of steroids.** Silylation was carried out directly in the tapered glass tubes. After adding 40 ml derivatization mixture containing MSTFA–TMIS– DTE (1000:2:2, v/v/w), the tubes were sealed tightly and heated at 60 ^{o}C for 40 min. Steroids with a side chain at C17 should be stored at 48 ^{o}C overnight to complete the reaction. The solution can be injected directly into the gas chromatograph (injection volume: 1ml) or the mixture evaporated under nitrogen and dissolved in 1.00 ml of hexane.
2. **GC/FID analyses** were performed on gas chromatograph with FID detector. To perform the analysis, follow the instructions included with the instrument. Oven temperatures were programmed as follows: initial temperature 140 $^{\circ}$C for 1 min, raised at 20 $^{\circ}$C min -1to 280°C and maintained at 280°C for 10 min. Sample injection volume was 2 μl.
3. In the analysis of the obtained spectrum of the steroid hormone mixture, the previously recorded spectra of reference substances should be used.
4. Dispose of solid and liquid byproducts in the appropriate waste containers. Wash all equipment and return it to its proper place.