INTRODUCTION

BASIC LABORATORY MATERIALS

HOW DO YOU SAY …?

HOW DO YOU SAY …?

Coat		**Scales**	
Gloves		**Volumetric flask**	
Safety goggles		**Stopper**	
Beaker		**Funnel**	
Stirring rod		**Burette**	
Dropper		**Wash bottle**	

HOW DO YOU SAY …?

Graduated pipette		**Spatula**	
Volumetric pipette		**Screw-top tube**	
Pro pipette		**Conical flask or Erlenmeyer flask**	
Micropipette		**Magnetic stirrer or Heater**	
Pipette tip		**Centrifuge**	
Graduated cylinder		**Centrifuge tubes**	
Test tubes		**Dispenser**	

THEORY DOSSIER
CHROMATOGRAPHIC METHODS

PRACTICALS 6 AND 7

1. BASIC NOTIONS ON CHROMATOGRAPHY

Main concept: Chromatography was first described in 1901. Today it includes a wide range of techniques that are commonly used to separate complex samples, thereby enabling the qualitative and quantitative analysis of those samples.

The components of the samples to be separated are generally distributed between two phases (or between one phase and one interface), one of which is mobile while the other is stationary. The mobile phase therefore advances in the direction in which the separation system advances, thus maintaining direct contact with the stationary phase, which, as its name suggests, is stable and does not move. When a sample is deposited in the chromatographic system, the mobile phase tends to advance the components of interest in the direction of its flow, while the stationary phase, which interacts with the components we wish to separate, delays their progress, especially those with which it has an enhanced chemical affinity (polar-apolar). The most retained components thus travel at a slower rate and are eluted later than the less retained ones.

Until now, your closest experience with chromatography on your Chemistry degree has been when performing thin-layer chromatography (TLC) in the Organic Chemistry Laboratory, where the separation of the components can normally be seen directly with a naked eye, usually thanks to the color of the separated components. In other types of chromatography, however, it is common to work with compounds that have no color or are present at much lower concentrations. The usefulness of chromatographic techniques therefore lies in detectors that are located at the output of the system, which make it possible to identify and quantify the components as they are eluted from the chromatograph.

In general, chromatographic separations are fast, reproducible, and selective. They allow us to separate highly complex mixtures in a short period of time and to distinguish between solutes or compounds with similar structural and physical properties that may not be differentiated by simpler separation techniques.

Types of chromatographic techniques: As mentioned above, many types of chromatographic techniques exist. Indeed, there are as many as there are needs that have arisen over time in relation to the separation of compounds. Several classifications have been proposed, including the design of the separation system (in a column or open supports), the operating mode (zonal or frontal elution), and the nature of the stationary phase. The aims of this dossier are to clarify the main concepts and aspects of chromatography, provide an overview of them, and focus on the chromatographic techniques we will use in the practical sessions for Analytical Chemistry Laboratory II. We will therefore focus only on the two types of chromatography we will use in the laboratory lessons on the nature of the stationary phase. We assume this content will be taught more thoroughly in Analytical Chemistry III.

The two main types of chromatography, depending on the nature of the stationary phase used, are:

- Gas chromatography by solution or gas-liquid distribution. The stationary phase is a thin liquid film (µm size) on a solid support. The separation of solutes is due to their balance between their gaseous state and their dissolution in the liquid part. The mobile phase is therefore an inert or relatively inert gas that serves as a vehicle for the analytes in the gaseous state but has no real affinity for them.

  This technique is used to separate any type of solute provided that it is volatile with temperatures below 350 °C, or that they can be easily converted into volatile derivatives before the technique is applied.

  There are numerous gas chromatography techniques depending on the type of detector used. The best-known ones are GC-FID (flame ionization detector, which we will use in the laboratory), GC-MS (mass spectrometry), and GC-ECD (electron capture detector).

Figure 1. External view of a gas chromatograph.

- Liquid chromatography by absorption or liquid-liquid distribution. The stationary phase is a liquid phase that coats a solid support, as with gas chromatography, and the separation of solutes in this case is a product of their balance between the two liquid phases in the system. Liquid chromatography usually achieves very high efficiencies and is therefore widely used. The best-known and most common technique is High-Performance Liquid Chromatography (HPLC), which is widely used to identify and quantify all types of solutes, especially those with non-volatile properties and for which gas chromatography cannot be used.

Figure 2. External view of a liquid chromatograph.

- Other types of chromatography, depending on the nature of the stationary phase, are ion-exchange chromatography and liquid exclusion chromatography, but these are not among the specific objectives for this course.

Some parameters to bear in mind when considering chromatographic separation are:

- The type of elution. How the compounds are eluted is a fundamental parameter. The elution of the compounds can be made more precise by varying the composition of the mobile phase or other properties.

  In general, elution is considered either isocratic (with no changes in working conditions during elution) or gradient. When we wish to separate solutes with very dissimilar properties, it is impossible for all of them to elute separately from the chromatographic system in an optimal time zone if we use isocratic elution. In such cases, the working conditions are changed gradually during elution by using a gradient.

  For example, in liquid chromatography, it is more common to use gradient elution than isocratic elution. The percentage of solvent in the mobile phase with the highest affinity for the compounds we wish to separate therefore changes progressively (it usually increases) during the separation. This means that the eluent force of the mobile phase gradually increases, and so the highly retained compounds in the stationary phase are eluted more rapidly. In reverse-phase liquid chromatography, the main principle of which is hydrophobicity, it is common to increase the percentage of organic solvent in the composition of the mobile phase. This increases the apolarity and, therefore, also the eluent strength.

  Similarly, in gas chromatography it is common to use a thermal gradient, which involves gradually increasing the temperature of the column. This

speeds up the elution of less volatile compounds or eases the separation of broadly similar solutes.

In summary, gradient elution, whether in liquid or gas form, enables the separation of both weakly and strongly retained compounds by the stationary phase in a reasonable period of time and in the same chromatogram.

- Detection mode. There are no universal detectors for any type of chromatography that are recommended for every type of application. Several detectors (UV, fluorescence, mass spectrometry, etc.) are commonly used today and each has a preferable field of application depending on the type of compound we wish to separate. However, regardless of the technique or physicochemical principle on which it is based, a detector should generally have little signal change (or drift), a low background signal (or noise) and a fast response. It should also be highly sensitive, easy to operate, robust and reliable.

2. HIGH-RESOLUTION LIQUID CHROMATOGRAPHY

There are three modalities of high-performance liquid chromatography depending on the polarity of the stationary phase:

- Reverse phase (RP-HPLC). The stationary phase is hydrophobic and the mobile phase is essentially polar (aqueous or hydro-organic). This is the modality we use in our laboratory lessons to analyze caffeine.
- Normal phase (NP-HPLC). The stationary phase is polar and the mobile phase is mainly hydrophobic.
- Hydrophobic interaction (HILIC). The stationary phase has a polar surface (similar to the normal phase) that is covered with a layer of adsorbed water. The mobile phase is a mixture of hydro-organic solvents (usually water and acetonitrile).

2.1. The HPLC chromatographic system

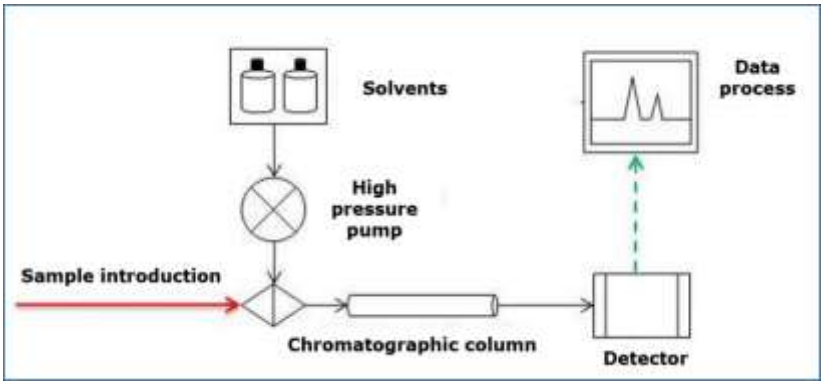

Figure 3. Main parts of an HPLC system.

Liquid chromatography equipment usually comprises the following elements or components:

a) (Between 1 and 4) solvent flasks and an on-line degasser to remove gases from the air that may be dissolved in the mobile phases. It is good that the mobile phases should be degassed since the solubility of the gases in the air depends on the proportions of the various solvents as well as on the ambient pressure and temperature. In non-degassed solutions, reduced solubility of the gases due to loss of pressure along the column causes bubbles to appear, which leads to false peaks in the chromatogram and an increase in background noise. As mentioned earlier, the mobile phases are usually de-gassed using on-line degassers: the mobile phase passes through a gas-permeable plastic tube located inside a reduced pressure chamber.

b) The pump module, which maintains a small but constant mobile phase flow through the system.

c) The injection module, which can be manual or automatic. In HPLC, this valve is sandwiched between the pump module and the columns. The essential part of the manual injection system is a 6-way, 2-position valve. The figure below shows the most important components of the manual injection valve.

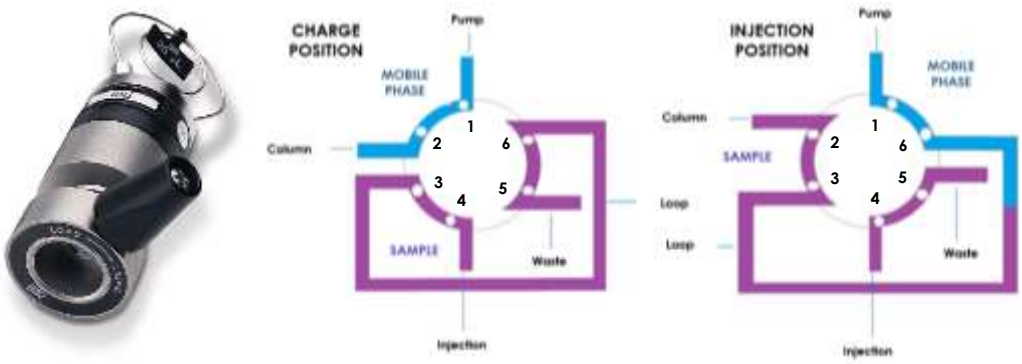

Figure 4. External (left) and internal (right) views of the manual injection system in liquid chromatography.

As we can see, the valve consists of an external body, which is fixed, and a central component, which can rotate and has two positions. The central body thus has channels that link the various ports or ways in pairs. Starting from the top and turning left, the intercommunicating ports are 1-2, 3-4, and 5-6 in one of two possible positions, and 2-3, 4-5, and 6-1 in the other position. For manual injection, two ports on the valve are connected by a steel tube of a well-known internal volume called a loop. In the loading position (left), a syringe is used to wash and load the loop with the sample. This operation is done with the open position. Turning the valve 60° clockwise switches to the injection position (right). In this position, the

contents of the loop are inserted into the pressure flow from the pump to the column.

d) The column. Columns in HPLC are usually short, i.e. typically 10 or 15 cm long with a maximum of 40 cm. They are made of stainless steel because the material must be able to withstand high pressures without becoming deformed.

e) The detector (spectrophotometric, fluorimetric, evaporative, electroanalytical, mass spectrometry, etc.) or two detectors one after the other given that the first one is a non-destructive detector.

f) The data acquisition and processing system. HPLC equipment usually has a modular design, which means that its modules can communicate with each other via a digital connection. It includes a data acquisition system, which is responsible for converting the signal received from the detector into an analytically interpretable signal.

2.2. The eluent force

As mentioned earlier, the eluent force is the ability of the mobile phase to elute the retained solutes into the stationary phase. In HPLC, it is extremely important to adjust the eluent force much more precisely (the result obtained with a 30% methanol mobile phase is not the same as that obtained with a 31% mobile phase). Moreover, gradient elution is widely and increasingly used in HPLC. Unlike for isocratic elution, a progressive increase in the eluent force of the mobile phase is applied in this case (e.g. the separation begins with 30% methanol and increases linearly to 80% in 30 min).

Eluent force is mainly used in the first stage of chromatographic methods to establish the composition the mobile phase must have to enable certain solutes with similar properties to separate efficiently. It is also used to express whether a particular change, such as increasing or decreasing the concentration of one solvent or changing one solvent for another, will increase or decrease the retention of the solutes of interest. It is logical to assume that solvents with a greater eluent strength lead to a greater change in retention.

The above chromatographic types have different elution forces, as we explain below:

- In RP-HPLC, water mixed with a miscible organic solvent, known as a "modifier", is used as a mobile phase. Common modifiers are eminently apolar organic solvents, such as acetonitrile, methanol and tetrahydrofuran. Moreover, since solutes can undergo acid-base reactions, a pH buffer must also be added to the aqueous component. In RP-HPLC, apolar solutes prefer the hydrophobic stationary phase, so elution occurs in order of decreasing polarity, i.e. the most hydrophilic solutes elute first, followed by the most hydrophobic ones. The eluent force of the mobile phase increases as its hydrophobicity increases: the more

similar the mobile phase to the stationary hydrophobic phase, the higher the eluent force obtained.

- In NP-HPLC, a hydrocarbon (usually heptane or cyclohexane) is used as the apolar mobile phase. To increase the eluent strength, these hydrocarbons can be mixed with increasing concentrations of miscible polar solvents. The main or most common modifiers are dichloromethane, chloroform, ethyl acetate, and isopropanol. In NP-HPLC, polar solutes show a preference for the polar stationary phase, so elution occurs in order of increasing polarity, i.e. the less polar solutes elute first, followed by the more polar ones. The eluent force of the mobile phase therefore increases as its polarity increases: the closer the properties of the mobile phase and stationary phase, the higher the eluent force.

- In HILIC, the stationary phase is the hydration layer of the solid support or the bounded phase. The mobile phase is usually acetonitrile in the presence of water, so the eluent force is increased by increasing the proportion of water in the mobile phase (in this case, the modifier is water). The solutes therefore elute in order of increasing polarity, as occurs with the normal phase. In fact, HILIC is a special type of normal-phase liquid chromatography.

In summary, the preference of a solute for a given mobile or stationary phase is determined by various forces involved in the mechanisms of solute-solvent interaction. It is therefore not an easy task. Various types of forces can exist in a solvent:

1. Interaction between permanent dipoles, between a permanent and an induced dipole, or between induced dipoles.
2. Electrostatic interactions (ionic repulsion and attraction).
3. Acid-base interactions by proton donation (acidity of the stationary phase).
4. Acid-base interactions by proton acceptance (basicity of the stationary phase).

Since different solutes exhibit different molecular properties, the eluent strength of a solvent or mixture of solvents cannot be measured by a single parameter that makes it valid for all types of solutes and all the variability that can occur. The eluent force is different for different solutes and different situations. For this reason, "eluent force" usually refers, mainly but not exclusively, to a polarity parameter that estimates the overall polarity of solvents, solutes and stationary phases in order to make a rapid analysis of the situation and the needs to carry out the separation efficiently.

2.3. Influence of column temperature

Regulating column temperature is not as important in HPLC as it is in GC. However, it is useful for improving separations. In fact, the temperature of the column is one parameter to be optimized when a chromatographic method is first developed.

The most common situation is to work in isothermal mode. Use of a temperature gradient during elution is usually avoided in order to avert longer waiting times before the initial temperature and mobile-stationary phase balance are reached between the acquisition of one chromatogram and the next. In some situations, however, it is mandatory to do so. In such cases, the most common working temperatures range from 5 to 80 °C.

Controlling the column temperature improves the reproducibility of the chromatograms, since fluctuations in the ambient laboratory temperature do not affect the separations that are taking place.

High temperatures during the separation have the following advantages:

- They decrease the viscosity of the mobile phase so that the flow is maintained with less pressure.
- They improve the solubility of certain solutes in the mobile phase.
- They modify the selectivity of the column, with changes in the order of elution of certain solutes.
- They increase efficiency at high flows because they increase the diffusion rate of the solutes.

The main drawback of high temperatures is that the lifetime of the column is considerably reduced.

2.4. Detectors

Most detectors used in liquid chromatography are spectrophotometric.

In spectrophotometry, the absorption of radiation in the UV-Vis region of the electromagnetic spectrum (from 200 to 800 nm) is measured. In this region, some inorganic ions and molecules and a large proportion of the organic ions and molecules absorb quite commonly. As we know, absorption occurs through the excitation of low-energy electrons, e.g. π and unpaired electrons. According to the well-known Lambert-Beer law, the signal is therefore proportional to the molar concentration of the absorbent solute.

Examples of spectrophotometric detectors are:

a) Spectrophotometric detectors with a Hg lamp. These are the simplest UV-Vis detectors and are equipped with a Hg lamp.

b) Variable-wavelength spectrophotometric detectors. Many spectrophotometric detectors are equipped with a deuterium lamp, which emits continuous radiation in the UV, and a tungsten filament lamp, which emits continuous radiation in the visible part of the spectrum. By using two lamps in this way, the whole range of UV-Vis is covered from 190 to 800 nm. The wavelength of the incident radiation is selected using a monochromator.

c) Diode array spectrophotometric detectors (DAD). Diode array detectors are able to detect the entire UV-Vis spectrum all at once. For this reason, the incident radiation is not dispersed before it reaches the sample. On the other hand, all the radiation emitted by the deuterium and tungsten lamps simultaneously affects the sample. For this reason the monochromator is located after the sample rather than before it as in the previous case.

Spectrophotometric detectors are the most widely used detectors in HPLC thanks to their easy operability, their low cost, the possibility of eluting with gradients, the wide range of organic compounds that are absorbed in the UV, and their sensitivity in many applications.

d) Unlike spectrophotometric detectors, HPLC uses other forms of detection, such as fluorimetric detection (which is useful for increasing sensitivity in compounds with fluorescent properties at very low concentrations), of refractive index, evaporative, amperometric, and coupled-mass spectrometers.

3. GAS CHROMATOGRAPHY

As we mentioned earlier, in gas chromatography the mobile phase is a gas and the stationary phase is the active surface of a solid or, more frequently, a liquid that coats a solid support either by deposition or chemical bonding (known as the linked phase). Depending on the nature of the stationary phase, the various types of gas chromatography are:

a) Gas-solid or adsorption chromatography, which is only used to separate compounds that are gases at room temperature, such as CO, CO_2, N_2, and NH_3, etc.

b) Gas-liquid or partition chromatography, which is used to separate all types of compounds. The only restriction is that the compounds must be sufficiently volatile at temperatures below 350 °C or able to form derivatives that are volatile at those temperatures. Some stationary phases do not decompose until roughly 450 °C, which enables a high-temperature GC to be used. This type is especially useful for separating very low volatile solutes, though these must be thermally stable.

Because of the much wider field of application for gas-liquid distribution chromatography, "gas chromatography" is understood to refer directly to that type of gas chromatography.

3.1. The mobile phase: the carrier gas

In gas chromatography, the gas used as the mobile phase is a simple carrier of analytes. There is no chemical interaction between the gas and the analytes because the nature of the gas does not influence the selectivity of the technique. However, the diffusivity of solutes is often higher if the gas is less viscous.

Greater diffusivity increases the rate of mass transfers within the gas, which improves efficiency when working at high flows. The best carrier gas is therefore H_2, followed by He and then N_2. Using O_2 or air is usually avoided in order to lower the risk of oxidation of the analytes or stationary phases.

Since H_2 is the least viscous of all the gases mentioned, it can greatly increase the flow and reduce the analysis time without losing efficiency. Other factors to consider are danger (H_2 is flammable), the requirements of the detector (e.g. with GC-MS it is recommended that He be used), price (He is better than N_2 but is much more expensive), and sustainability (e.g. He is a non-renewable resource since it is extracted from fossil fuels).

However, to increase efficiency and save time, the most widely used carrier gases in gas chromatography are H_2 and He.

3.2. Diagram of the instrument

The essential components of the gas chromatograph are illustrated below:

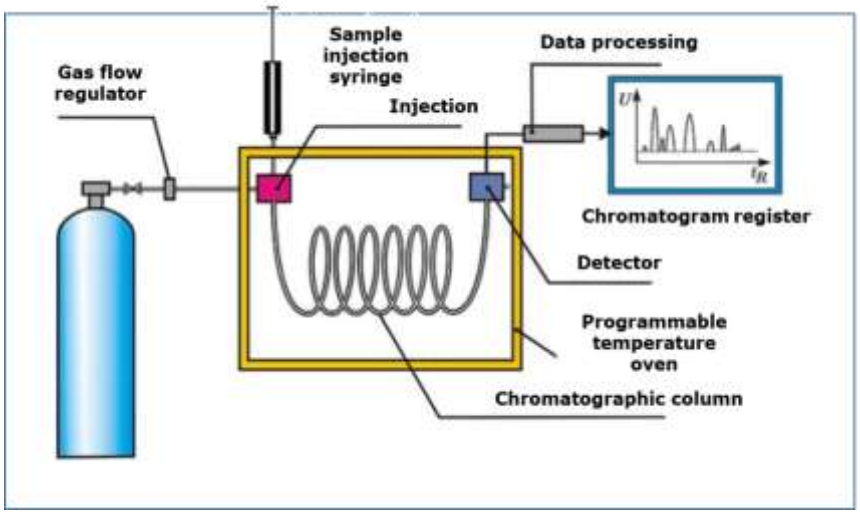

Figure 5. Main parts of a GC system.

a) Gas supply. He, N_2, air, and H_2 are commonly used depending on the detector. The gas can be supplied in the form of bottles or by in situ gas generators, since N_2 can be concentrated from the air and H_2 can be generated by the electrolysis of water. At the bottle or bullet outlet, a pressure reducer must be provided with two manometers, one to control the pressure remaining in the bullet and the other to act as a gas flow regulator at the inlet of the chromatograph.

b) Sample injection with independent temperature regulation. In the injector, the sample must be quickly vaporized in order to be dragged by the carrier gas into the column. The whole or part of the sample must be transferred to the column in a prompt and representative manner. A suitable speed is therefore needed for the sample to occupy a narrow strip at the beginning of the column so that efficiency can remain high. Vaporization of the sample is therefore considered a critical stage.

   Vaporization is also critical for preserving representativeness. The most volatile components in the sample vaporize before the heaviest and least volatile ones. Heavy components, which take longer to enter the column, obtain deformed and low-efficiency peaks. If the injection is stopped before the whole sample has been vaporized, it is unrepresentative (a lower proportion of heavy components is represented than the sample actually has). This phenomenon, known as mass discrimination, produces chromatograms with high sensitivity for light components and depressed sensitivity for heavier ones. To achieve a fast and representative injection, the following techniques are therefore used:

   1. Injection in *split* mode. Here, the sample is volatilized in a chamber, where the vapors are instantly mixed. When injected, the carrier gas flow is stopped for a few seconds in order to mix the vapors produced. The flow then increases sharply when the outlet is opened. Only a small fraction of the vapors is able to enter the column in this way. The rest (approximately 99%) is rejected. This injection technique eliminates the queues that would occur if remnants of the volatilized sample continued to enter the column.

   2. Injection in *splitless* mode. As in the previous case, once the sample is injected, the flow of the carrier gas is stopped and the sample is injected inside the cannula while always keeping the outlet closed. In this way, almost all the vapors produced enter the column. However, some sample is also lost during purging to remove the last remnants of the sample.

   3. Direct injection into the column. In this case, no cannula is used but a longer needle is needed so that its end is slightly inserted into the column. As all the steam enters the column, mass discrimination is not carried out. However, it should be noted that there is a risk of damaging the column, especially if the sample contains non-volatile components that will be permanently deposited. This type of injection is therefore reserved for high-boiling solutes, which evaporate very slowly in the cannula, and for thermally unstable solutes, which can suffer degradation when in contact with the materials in the cannula.

In all cases, the samples are injected in liquid form either manually or with an automatic injector. A Hamilton syringe that punctures through a septum in the chromatographic system is used. These syringes are designed to accurately inject very small volumes of liquid samples (between 1-50 µL).

c) The column, which can be a filled column or a capillary column. The most common stationary phases in gas chromatography are high molecular weight polymers. However, several other types exist:

   1. Apolar phases. These retain solutes by hydrophobic or van der Waals forces. They are used to separate apolar solutes or polar solutes that have an important apolar component in their structure.
   2. Polarizable phases. These retain polarizable solutes by forming induced dipoles.
   3. Polar phases. These contain permanent dipoles and retain polar or polarizable solutes such as alcohols, ketones, ethers, esters and amines, etc.
   4. Very polar phases. These retain solutes via an attraction between permanent dipoles and/or the formation of hydrogen bonds. The solutes must have at least one highly polar functional group.

d) The temperature oven with independent temperature control. The maximum operating temperature (350-450 °C) is limited by the risk of thermal decomposition of the stationary phase and, in unlinked phases, also by loss of viscosity of the phase. However, the field of application for gas chromatography can be extended to many non-volatile compounds by conducting prior solute derivatization reactions.

   The minimum working temperature is the melting point of the stationary phase. A solid stationary phase cannot retain solutes since these will not be dissolved. The minimum working temperature is also limited by the excessive increase in viscosity experienced by certain polymeric phases at low temperatures. With excess viscosity, mass transfer is extremely slow, so the loss in efficiency is low.

e) The detector, or detectors if a first non-destructive detector is used followed by another detector in series.

f) Rotameters or flow meters. These are located at the gas inlet tube and the column outlet.

g) A computer system for acquiring, processing and presenting data.

3.3. Order of elution of solutes: boiling points and affinity

If the stationary phase is apolar, retention can only occur by van der Waals forces. These interactions are called "non-specific" to distinguish them from the stronger or "specific" interactions that take place between polar functional groups. In the absence of specific interactions, the solutes leave the column in ascending order of their boiling points. If the stationary phase is polar or polarizable, specific interactions will also occur with solutes that have polar or polarizable functional groups. In this case, the order of elution will be determined by the following two rules:

a) The boiling point rule. Members of the same homologous series of compounds have similar chemical properties, so the stationary phase cannot differentiate between them based on specific interactions. Members of the same homologous series are differentiated based on their volatility, i.e. in ascending order of their boiling points.

b) The affinity for the stationary phase rule. Solutes of different homologous series are differentiated based on their affinity for the stationary phase. The greater the interaction of the solute with the stationary phase, the greater the retention.

3.4. Gas chromatography at a programmed temperature

It is not possible to simultaneously separate solutes with very different boiling points. If a low temperature is chosen, the low boiling point solutes will separate but those with higher boiling points will never leave the column. Similarly, if a high temperature is chosen, the high boiling point solutes will separate properly but the low boiling point solutes will appear at retention times close to the beginning of the chromatogram and will not separate. The same problem occurs when compounds of any type that have very different polarities are chromatographed.

The problem is solved by a temperature gradient, i.e. by working at a programmed temperature. The column temperature program runs immediately after each sample is injected and usually starts at a relatively low constant temperature. After a few minutes, the temperature begins to rise linearly. This rise may be constant or have various sections with different gradients. This is followed by a few minutes at high temperature in order to facilitate the elution of the heavier components of the sample. Finally, the process returns to its initial temperature before another sample is injected.

A lower initial temperature than the boiling points of many solutes is often used. This technique is called **cold trapping**. In this way, the solutes are retained at the head of the column, dissolved in the stationary phase, and converted into the gas phase as they reach their respective boiling points. As the temperature continues to rise, the solutes accelerate and elute rapidly.

3.5. Detectors

The detector most commonly used in gas chromatography is the flame ionization detector (FID). This is the detector used to determine phenolic compounds.

With FID, N_2 is usually used as a carrier gas because it is less dangerous than H_2 and cheaper than He. At the outlet of the column, the gas mixes with small flows of H_2 and air to keep the flame on. The flame burns inside a ring that is connected to the current. At the same time, the tip of the burner is also connected to a higher potential in comparison to the mentioned ring. In the absence of charge carriers, the potential difference is 0. The current can pass through the system only if ions or electrons appear in the gas (within the flame). When H_2 burns it does not produce ions, so the H_2 flame does not conduct electricity.

The sensitivity of the FID is proportional to the number of oxidizable carbon atoms the compound has to separate. The response decreases with increasing substitution with halogens, amines, and oxhydryls and the contribution of highly oxidized carbons is zero.

The FID detector has both advantages and disadvantages. For compounds with oxidizable carbons, very low detection limits are reached. Also, it has a very wide linear range and is stable, simple, and robust. However, since it is a destructive detector, it cannot be coupled to a mass spectrometer.

The designs of several other detectors are similar to that of the FID. Modifications can make the detector more sensitive, or even more specific, for measuring certain chemical species, for example. These detectors include the thermionic or nitrogen-phosphorus emission detector and the flame of the phosphorus-sulfur photometer.

4. LITERATURE

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PRACTICAL 6: DETERMINATION OF CAFFEINE BY HPLC

- How does an increase in the acetonitrile concentration of the mobile phase affect the retention time of the studied analytes?
- How does modifying the PH in the mobile phase affect the retention time of the studied analytes?
- How does modifying the mobile phase flow affect the retention time of the studied analytes?
- How does modifying the wavelength affect the chromatograms obtained?
- Why do we de-gas the mobile phase before introducing it into the chromatographic system?

PRACTICAL 7: DETERMINATION OF PHENOLS BY GC

- Why is the urine sample heated in HCl?
- What are the extraction cartridges made of? Could the solid-phase extraction be performed at basic pH in this case?
- What steps is the solid-phase extraction composed of?
- Why do I have to activate the extraction cartridges?
- How would the pre-concentration factor change if we changed the sample or extraction volumes, or if the extraction yield were different?
- What are the advantages of using a temperature program in gas chromatography?
- By considering the structure of the molecules and their properties, explain the retention times obtained.
- What is the internal standard for? What compound do we use as an internal standard in this case?

THEORY DOSSIER
ELECTROANALYTICAL METHODS

PRACTICALS 8, 9 AND 10

1. BASIC NOTIONS ON ELECTROANALYTICAL METHODS

1.1. Electrochemical cells

Anodes and cathodes. An anode is an electrode (a solid conductor, usually a metal) where an oxidation process takes place. A cathode, on the other hand, is an electrode where a reduction process takes place. In short, a cathode is an electron absorber whereas an anode is an electron source. Therefore, at least one species loses electrons on the surface of an anode while at least one chemical species gains electrons on the surface of a cathode.

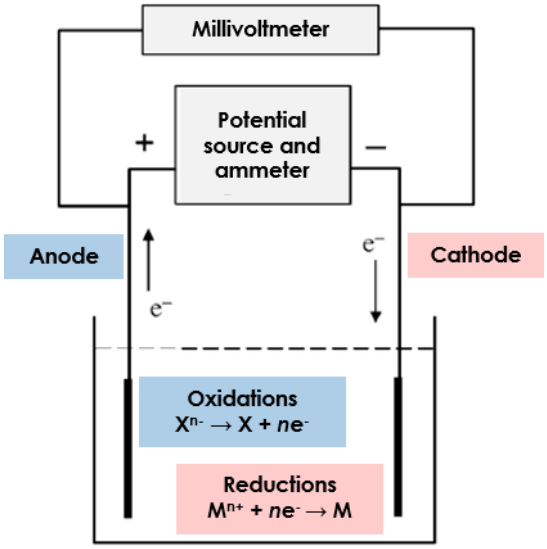

Figure 1. Diagram of an electrochemical cell.

Galvanic cells and electrolytic cells. An electrochemical cell is a circuit made up of at least two electrodes that are in electrical contact through a solution. The solution must be ionic, i.e. it must contain ions, which are the carriers of the current. Dissolved ionized substances are called electrolytes.

The cell is called galvanic when it is used to produce an electric current from a spontaneous redox reaction within it and is called electrolytic when an imposed potential (in the opposite direction of the galvanic cell) and an externally generated current are used to develop a non-spontaneous redox reaction. There is therefore no difference between a galvanic cell and an electrolytic cell from the external point of view, since both can be constructed with the same electrodes submerged in the same solution. The only difference between them is the way the cell is used, i.e. to produce energy if it is galvanic or to consume energy if it is electrolytic. An electrolytic cell can be constructed with both electrodes submerged in the same solution or in different solutions.

If the electrodes that form the electrochemical cell are submerged in different solutions, a third solution is needed to close the circuit. This is the salt bridge, which allows the passage of ions, i.e. the passage of current, from one solution to the other. Ions are what carry current through the solution, just as electrons are what carry current in metal. Another advantage of the salt bridge is that it keeps the

anodic and cathodic reactions separate, thus preventing the reaction products from mixing, which in turn enables the reactions to be completed safely.

The best-known assembly of a galvanic cell is the **Daniel Cell**. This consists of a copper electrode (which acts as a cathode) and a zinc electrode (which acts as an anode). Zinc is more likely than copper to dissolve in the solution of its ions. As a result, zinc oxidizes and releases electrons. Through the zinc electrode and the external circuit, the electrons that are released end up on the surface of the copper. Cu^{2+} ions that are close to the surface of the electrode use these electrons to reduce themselves, thus becoming part of the metal. The process soon results in excess cations in the zinc solution and a defect in the copper solution. Note that the process would stop if there were no possibility of transporting the ions across the salt bridge. The chloride (Cl^-) and potassium (K^+) ions that exist through the salt bridge maintain ionic balance, i.e. complete electrical neutrality in the solutions. The overall reaction of the Daniel Cell is:

$$\mathbf{Zn(s) + Cu^{2+} \rightarrow Zn^{2+} + Cu(s)}$$

For this reaction to advance, the current must also be allowed to pass through the external circuit. Millivoltmeters and/or ammeters are usually used for this purpose. Assuming that the circuit is closed and the resistance of an ammeter is negligible, electrons pass through the circuit, and the reaction proceeds. The current decreases slowly until it reaches zero. At this point, the system will have reached equilibrium: the excess of Zn^{2+} ions in the cathode and the shortage of Cu^{2+} ions in the anode balances out the greater tendency of zinc to dissolve, and the reaction stops.

However, if the ammeter or the millivoltmeter is replaced with a source of direct current and a sufficient voltage is applied (positive for the Cu and negative for the Zn), the same cell will function as an electrolytic cell, i.e. the overall reaction will be the opposite:

$$\mathbf{Cu(s) + Zn^{2+} \rightarrow Cu^{2+} + Zn(s)}$$

In this case, the copper electrode forms an anode (oxidation, it dissolves) and the mass of the zinc electrode increases (reduction, it precipitates) in the cathode.

1.2. The electrochemical cell for analytical measurements

A typical electroanalytical cell usually consists of three electrodes: an indicator electrode, a reference electrode, and an auxiliary electrode.

- **The indicator electrode** is also known as the working electrode and is sensitive to the concentration of the analyte.
- **The reference electrode** provides a precisely known potential, or at least a potential of constant value. To ensure that the concentration of any chemical species that maintains its potential does not change or is not exhausted, the current passing through the reference electrode is made to be very small. This is done by connecting the reference electrode to a

millivoltmeter with a high internal impedance (i.e. with high resistance to the passage of current). Note that the reference electrode is usually separated from the sample solution via a salt bridge (e.g. a tube with a KCl solution) that is part of the same device.

- **The auxiliary electrode** is also known as the counter electrode and is responsible for withstanding the current when electrolysis occurs. It is usually large in order to reduce resistance and is connected to the current source to produce the electrolysis. The auxiliary electrode is needed to withstand the opposite redox half-reaction to that which occurs at the indicator electrode (oxidation or reduction). The current can pass through the auxiliary electrode, thus freeing the reference electrode from performing this task.

Not every electroanalytical cell has an auxiliary electrode (only those in which it is necessary to produce electrolysis do). In potentiometry, for example, the current passing through the electrodes is negligible and there is no interest in measuring it. Cells for potentiometric measurements therefore have only indicator and reference electrodes.

On the other hand, the auxiliary electrode is needed in coulometries, voltammetries, and amperometries in which a non-negligible current passes through the indicator electrode. With these techniques, a potential difference is applied between the working electrode and the reference electrode, and the current passing through the indicator electrode is measured. The circuit is closed on both sides (reference and auxiliary). However, because of the high resistance in the reference electrode, the auxiliary electrode is needed to enable almost all the current to pass through it.

1.3. Electrochemical reversibility and irreversibility

In chemical equilibrium, reversibility refers to the speed with which direct and inverse reactions take place. In electrochemistry, however, the concepts of reversibility and irreversibility are different. Reversibility applies mainly to redox half-reactions considered separately: one may be reversible while the other is irreversible.

A reaction is reversible when it is fast enough to make the influence of its speed on the current go unnoticed in the conditions used to carry out the measurements. Sometimes we can also talk about reversible and irreversible redox processes. A redox process is reversible when the two half-reactions (oxidation and reduction) are fast enough to make the influence of velocity on the current go unnoticed in the conditions used to carry out the measurements.

Electrochemical reversibility occurs when the species that is oxidized or reduced is chemically stable, so the rate of electron transference is expected to be very fast. A half-reaction can also be reversible under certain working conditions and become irreversible under others (e.g. lower temperature, etc.). If the half-reaction has zero velocity, i.e. it cannot occur, it is called irreversible.

1.4. Classification of electroanalytical techniques

a) **Techniques in the solution**. These measure phenomena that take place in the solution. For this reason, no chemical reactions are carried out at the interface.
b) **Techniques in the interface**. These measure phenomena that take place at the interface of the electrodes and may be:

- Static (current I=0): potentiometry and potentiometric standardizations.
- Dynamic (current I≠0): voltammetry, coulometry, and electrogravimetry.

2. DIFFERENTIAL PULSE VOLTAMMETRY

The basis of voltammetry is measurement of the current that flows through the cell to which a potential sweep is applied, thus obtaining a current-potential graphic. This is called a voltammogram, which may be considered the electrochemical equivalent of the spectrum in spectroscopic techniques or of the chromatogram in chromatography. Voltammograms provide qualitative and quantitative information about the electroactive components of the sample, i.e. the species that carry the current due to their participation in oxidation and reduction processes.

As we have mentioned, this technique uses a cell with three electrodes. Specifically, these are the working or indicator electrode (in our case, a graphite-rotating electrode), the reference electrode (Ag/AgCl), and an auxiliary electrode or counter electrode (Pt). The potential difference between the working electrode and the reference electrode varies over time, so the current that flows between the first and the auxiliary electrode is measured. The reference electrode is constructed in series with a high impedance circuit, precisely to divert almost all the current through the counter electrode. This prevents exhaustion of the reference electrode and reduces the ohmic drop to a negligible level.

The indicator electrode can be made of Pt, Hg, Au, Ag, Sn, W, or C (which is a vitrified or pyrolytic graphite paste). Solid electrodes are usually constructed in the form of a disk and are usually mounted (embedded) at the end of a support or rotating device. Carbon paste electrodes are made by filling the cavity at the end of the support with a graphite carbon paste bounded with viscous oil.

The main problem with solid electrodes is surface poisoning, since a deposition of insoluble species formed by electrolysis or simply collected from the solution may occur. As a result, a layer forms that alters the properties of the surface of the electrode. Another poisoning mechanism is oxidation of the same material present in the electrode (e.g. gold, which forms Au_2O_3). There are two solutions to the problem of poisoning, i.e. mechanically polishing the surface with an abrasive (e.g. a paper impregnated with an abrasive powder, which is usually

Al_2O_3) and, where possible, also applying a potential that removes unwanted substances.

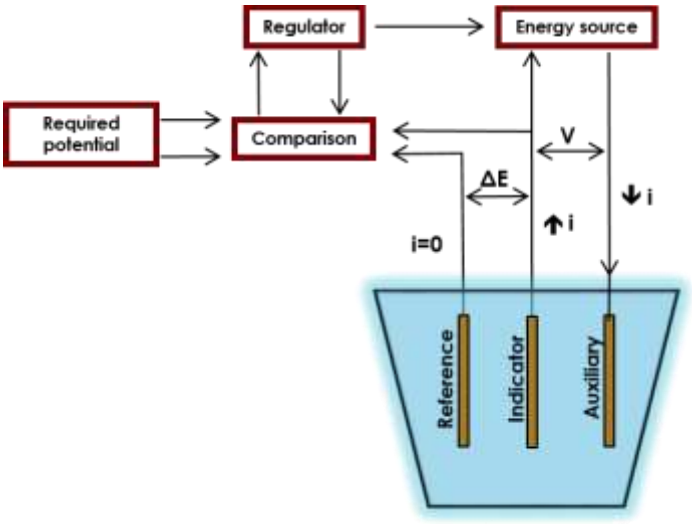

Figure 2. A voltammetry electrochemical cell (in our case, there is no nitrogen current because the O_2 in the solution does not prevent determination).

An analyte oxidizing on the surface of the working electrode causes a stream of electrons to pass from the solution to the electrode. This current would not occur without the corresponding reduction of another chemical species in the auxiliary electrode. We are usually only interested in one of the two half-reactions (oxidation and reduction) of a given species. We therefore try to develop this process in the working electrode and let the opposite half-reaction (which we are not interested in) take place in the counter electrode.

To select the suitable voltammetry technique, other factors are taken into account, such as the level of concentration of the analyte and the environment or location of the sample. Amperometric sensors are especially used in flow systems and whenever the measurement requires a certain speed, as in clinical analysis. On the other hand, pulse polarography or any of its variants is used to analyze a wide variety of inorganic and organic analytes at low concentrations. Importantly, some techniques reach sufficiently low detection limits without the waiting time needed by other techniques.

2.1. How does differential pulse voltammetry work?

A widely used technique in voltammetry is pulse polarography in both the normal and differential mode. With pulse techniques, the potential is not swept away by a simple ramp but is increased in strokes, or pulses. These are superimposed onto a linear ramp that generates a current that is measured in order to quantify the analytes. In this course we use differential pulse polarography, where, after each potential pulse or rise, the new potential is held for a few milliseconds. This is immediately followed by a "step back", i.e. a small drop in potential, which creates a capacitive current in the opposite direction to that created by the earlier increase. This leads to neutralization of the capacitive current, which

interferes with the measurement, which falls much faster to zero. For this reason, the current can be sampled longer within each cycle and with less residual current. It is also possible to reduce the duration of the cycles and therefore obtain more information for each potential sweep.

In pulse polarography, the sweep is characterized by various parameters (see *Figure 3*), including the duration of the pulses (t_p), the duration of the waiting time between pulses, the total duration of the cycle, the waiting time, the height of the pulses (ΔEp), and the height of the step back that occurs at the end of each pulse (ΔE_s). Numerous variables can therefore improve the characteristics of the voltammograms obtained, including better detection limits and increased selectivity towards other species that also oxidize or reduce themselves.

To further reduce the influence of the residual current on the signal, the derivative of the intensity-potential curve is automatically calculated. This helps to achieve a further improvement in the signal-to-noise ratio, and a graph representing the current derivative with respect to the potential is obtained. A differential pulse polarogram therefore shows peaks instead of waves. Each peak corresponds to a point that represents the maximum slope of the wave, while the height of the peaks is proportional to the concentration of the analytes in the solution. Another way to remove some of the residual current due to impurities is to prepare the sample well. The most common and most important interferent is dissolved oxygen, which shows two highly characteristic reduction waves of the same height: from O_2 to peroxide (-0.1 V) and from peroxide to water (-1.15 V). Dissolved oxygen is removed by purging the solution with oxygen-free nitrogen. To remove traces of oxygen that contaminate the nitrogen, the gas is previously passed through a strongly reducing solution. In fact, one application of voltammetry is in the determination of dissolved oxygen.

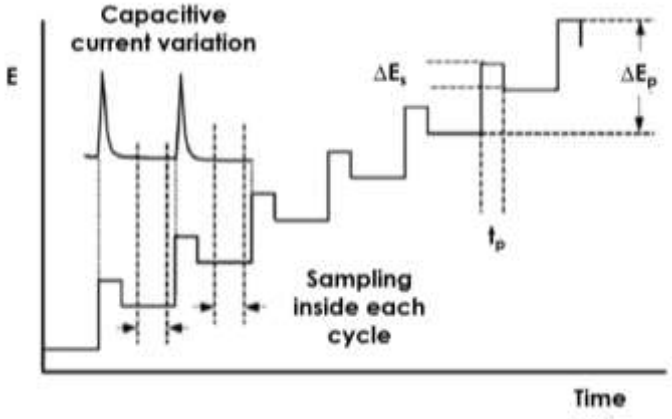

Figure 3. The sweep of potential in pulse polarography.

The biggest advantage of differential techniques is that they provide information in the form of peaks rather than waves, so they are better able to distinguish between analytes that oxidize or reduce with half-wave potentials ($E_{1/2}$) that are very close to each other. Typical applications of these techniques are the trace determination of numerous chemical species, such as metal cations, inorganic anions, and organic substances with electronically active groups, e.g. carbonyls (aldehydes, ketones, quinones), carboxylic acids, peroxides, halogenated derivatives, double conjugated bonds, nitro groups and amino groups, etc.

3. POTENTIOMETRY

In direct potentiometry, the concentration of the analytes is related to the potential the analytes develop on an indicator electrode. The potential of the indicator electrode is measured in relation to that of a reference electrode, since the indicator and reference electrodes are connected in series with a high impedance millivoltmeter. The potential of an electrode is therefore a measure of the "pressure" exerted by electrons to either leave the surface of the electrode or to "land" on it. The chemical species present in the solution therefore tend to oxidize or reduce and the potential of an isolated electrode cannot be measured. What we actually measure is the potential difference between two electrodes. If one of these is the reference electrode, and its potential is therefore known, a millivoltmeter informs us of the potential in the indicator electrode.

As we have mentioned, potentiometric cells are constructed so that one of the semi-cells provides a reference potential while the other can be used to measure the concentration of the analyte. To reach a compromise, the reference electrode is always considered the anode, i.e. the indicator the cathode. The most important reference electrodes include the standard hydrogen electrode, which also serves to mark zero on the normal potential scale, the calomel or saturated calomel electrode, and the Ag/AgCl electrode, which is the most common type of electrode.

The Ag/AgCl electrode is constructed with an Ag wire immersed in a KCl solution. When an Ag wire is immersed in diluted HCl, the surface of the metal is quickly covered with a layer of AgCl so that the wire can operate immediately as a reference electrode. The AgCl-coated wire is in turn immersed in a solution of KCl saturated in AgCl. Although the saturation concentration in this salt is very small, it prevents any loss of the metallic Ag of the electrode over time. The electrode also has a hole in order to fill the salt bridge with a saturated AgCl solution that can be saturated with KCl or not. The KCl solution functions as a salt bridge. The advantage of working with a KCl-saturated electrode is that it ensures proper functioning thanks to the presence of crystals at the bottom of the tube. When a saturated electrode is used, the hole can also be used to insert KCl crystals if these become scarce.

Various indicator electrodes exist, including ion-selective electrodes, where the obtained potential mainly depends on a particular ion. Ideally, they should be sensitive to only one ion. However, real electrodes are also sensitive to other ions that are considered their interferents. These include the glass electrode, polycrystalline membrane electrodes, and enzymatic electrodes, etc.

The selective fluoride electrode (LaF_3 single-crystal fluoride electrode) is the only electrode that has been successfully constructed with the single crystal of an insoluble salt. This electrode has excellent operating features. The membrane is a trivalent lanthanum fluoride crystal (LaF_3) with a divalent europium fluoride impurity (EuF_2). The presence of Eu^{2+} in the crystal leaves holes (where there is an Eu ion, there is a lack of fluoride for LaF_3) that enable the movement of charges (F^- ions) through the crystal. This gives the crystal enough conductivity to transmit the potential from one side to the other. The fluoride electrode is used to determine fluoride and cations that form salts which are insoluble in fluoride (such as Al^{3+}).

The operation is not conducted by ion exchange but by the potential difference (at current I=0) between the interior and exterior components of the electrode. This depends on the activities of the ion F^- in the internal and external solutions of the electrode. On both the internal and external surfaces, the following equilibrium takes place:

$$\mathbf{LaF_3\,(solid) \leftrightarrows LaF_2^+\,(solid) + F^-\,(solution)}$$

The fluoride electrode is the only one that is virtually interference-free. However, it cannot be used below pH 4 or above pH 8. Below pH 4, fluoride is protonated and forms hydrofluoric acid (HF). Above pH 8, the hydroxyl ion interferes strongly due to the tendency of lanthanum to form lanthanum hydroxide ($La(OH)_3$).

Note that the electrodes are part of a potentiometric cell that measures activity but not concentration. Great care must therefore be taken with the stability of the activity in the various solutions (both the standards and the samples measured) in order to easily relate the activity measured to the concentration of the analytes. One method widely used to maintain the stability of the activity in different solutions is TISAB. This method, which is applied to diluted and low ionic samples, involves adding a concentrated ionic solution (always at the same concentration) to samples and standards. This reduces the differences in the matrix between samples and standards to negligible values because the activity coefficients mainly depend on the electrolyte that is added at a high concentration. Another name for this method is "ionic strength" adjustment.

4. LITERATURE

- RAMIS-RAMOS, G. *Apuntes Química Analítica III*, Universitat de València, 2013.

PRACTICAL 8: DETERMINATION OF FLUORIDE IN TOOTHPASTE

- What type of electroanalytical technique are we using? What are we going to measure? What are the units of the signal obtained?
- What does our measuring device comprise? How many electrodes are there? What is each electrode used for?
- What is TISAB? Why do we use it?
- How does pH affect the measurement and why?
- How does the ionic strength affect the measurement and why?
- How does the presence of other cations affect the measurements and why?
- What data are represented to obtain the calibration curve?

PRACTICAL 9: ELECTROGRAVIMETRIC DETERMINATION OF COPPER

- What does the device that is used look like? What are its components?
- What is HNO_3 used for?
- What analytical technique are we using?
- What is nitrate for?
- What is urea for?
- Where is the copper deposited?
- How do we check whether the electrodeposition has been quantitative?

PRACTICAL 10: VOLTAMMETRIC DETERMINATION OF TOCOPHEROLS

- What calibration method are we using?
- What type of electroanalytical technique are we using? What are we going to measure? What are the units of the signal that we will obtain?
- What does our measuring device comprise? How many electrodes are there? What is each electrode used for?
- How many peaks should we observe in the voltammograms?
- What solution do we add to the oil sample with tocopherols to conduct the extraction? What is it composed of?