الجمهورية الجزائرية الديمقراطية الشعبية
وزارة التعليم العالي والبحث العلمي

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DEPARTMENT OF BIOCHEMISTRY

Practical Work:

Experimental Pharmacotoxicology Of active substances

***Level*: 1st Year Master (LMD)**

Applied Biochemistry

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Practical Work

Preface

FOREWORD

The practical work presented in this handout is intended for students in **Master 1 Applied Biochemistry**. They concern **experimental pharmacotoxicology of active substances**, important operations for the evaluation of their performance, as soon as they leave the manufacturing chain on an industrial scale.

This handwritten copy described in **eight PW** some basics of*analysis techniques chemicals*for the control of pharmaceutical products. They will allow students to become familiar with bench work and acquire these methods.

Each TP statement corresponds to a session **of two hour**. It includes adetailed operating mode and practical or theoretical questions allowing the interpretation of the experimental facts observed.

Particular emphasis was placed on the safety conditions necessary for the properprogress of a practical work session, in accordance with current regulations and on all glassware used in these different experimental achievements.

How to write a report

How to write a report

For practical work in chemistry, a practical work booklet is distributed to students in which the experimental protocols to be followed are precisely detailed. A practical work report is requested from students in order to evaluate the student's level of understanding and analysis.

Teachers expect students to show through their report that they have not only demonstrated rigorous work during the practical work through the quality of the results obtained but also that they have a good sense of analysis and interpretation of their results. The goal is therefore not to copy what is written in the practical work booklet!! Here are some tips to follow to write an effective report.

A practical work report should, in general, be composed of 5 distinct parts:

1. **Cover page**

2. **Introduction**

   Quickly prepare (about 10 lines) the principle and the aim of the practical work by giving the chemical concept covered.

3. **Protocol and results**

   Present the graphs (with title, chosen scale, units and name of the axes), the tables (with title), the calculations of the requested quantities (with units) with their associated uncertainties. Also present the protocols that do not appear in the brochure (dilution, etc.)

4. **Analysis of results and discussion**

   Interpret curves and tables. Comment on the results using a theoretical approach.

5. **Conclusion**

   Briefly summarize the results and comments obtained.

1. GENERAL INFORMATION

1.1. Laboratory safety

These few paragraphs are intended to remind you of some safety instructions. Learning them is mandatory.

- **Health Hazards**

It is absolutely necessary to prevent chemicals from entering the body, because they are always more or less toxic or dangerous. In their immediate action,one can be only inconvenienced (tingling when inhaling sulfur dioxide for example), seriously affected (inhalation of chlorine can cause pulmonary edema and allergic reactions) or fatally poisoned (arsenic or cadmium for example).

At the entrance to each laboratory, a poster indicates: The name of the laboratoryand its contact details, safety instructions, warnings and the dangers of chemical products (**Figure 1**).

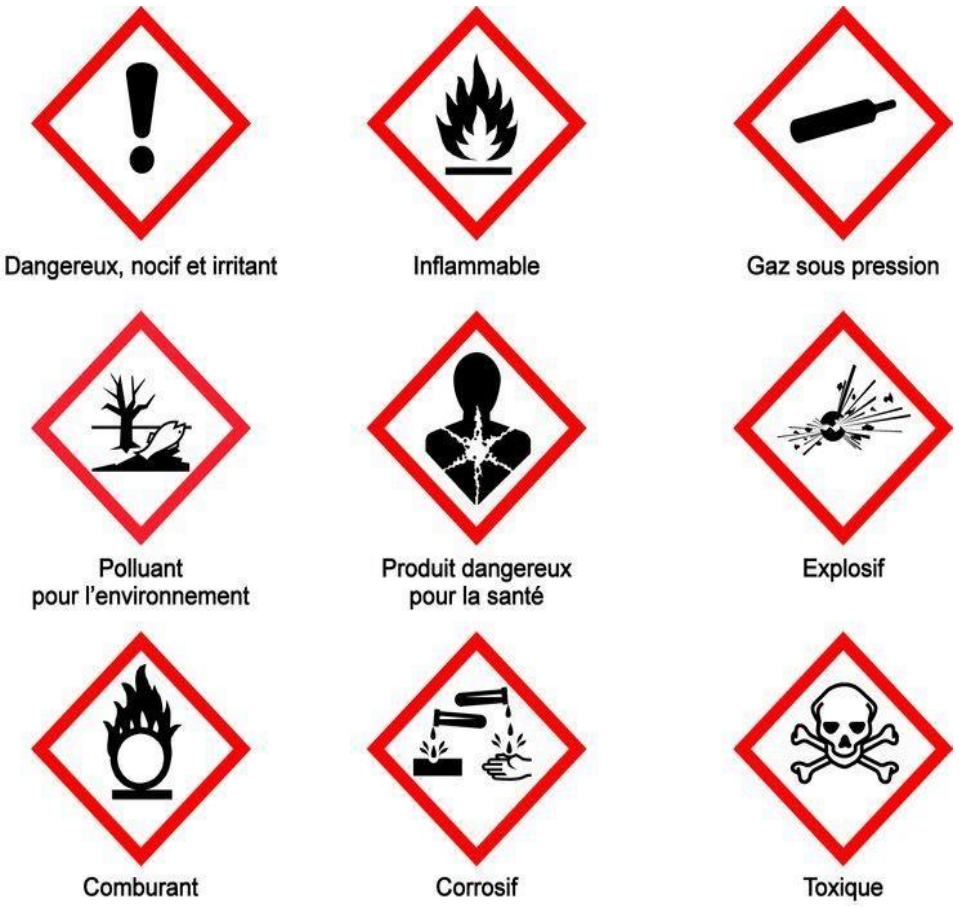

Figure 1. Risks of chemicals

Chemicals have four routes of invasion into the body: the skin, mucousmembranes, lungs and digestive system.

- **Behavior**

It is therefore forbidden to drink, eat or smoke in chemistry laboratories: to prevent the risk of ingesting chemicals. It will also be necessary to get into the habit of washing your hands when leaving. Caustic products (bromine, strong mineral acids, strong bases, powerful oxidants, etc.), compounds that easily penetrate the skin (aromatic amines, nitrate derivatives, etc.) will be handled with gloves and especially safety glasses.

- ✓ Wearing safety glasses is mandatory whenever you have to handle or see someone handling acid or basic solutions with a concentration greater than 0.05 M.

- ✓ Wearing a flame-resistant coat is mandatory at all times. It obviously protects street clothing from various types of dirt (dyes, acid perforations, etc.) but especially from fire and from impregnation of clothing close to the body by corrosive products.

- ✓ Finally, while you have to take care of yourself, it is also very important to think aboutprotecting others through a responsible attitude. You should avoid cluttering your workbench: a handout, a pencil, a notebook and a calculator are usually enough. Any uncontrolled movement or disorderly movement is a cause of accident in a laboratory. The greatest calm must be maintained in all circumstances.

In case of accident

- ✓ Immediately warn your immediate surroundings**.**

- ✓ You will not be handling any deadly products, and the most significant risksto you are eye burns, body or facial burns, and possibly allergic shock.

- ✓ Absolutely remain calm and avoid large gestures and other disorderly movements.

The most effective countermeasure is to **Rinse thoroughly with water as quickly as possible**. In all cases, it is necessary to prevent the accident, and in case of burns rinse abundantly with tap water, especially not with purified water. Do not hesitate to **use safety showers immediately**. To rinse the eye only, rinse your hands then run water into the palm of your hand, dipping your open eye into it, slightly to the side.

1.2. Laboratory

- **Products**

Any chemical product is potentially toxic; therefore, avoid tasting, smelling or touching it. Be wary of fine powder products that can be inhaled. The risks associated with improper use of all the chemical substances that you will be required to handle are numerous and varied. You must always be vigilant. All commercial bottles have a label symbolizing the main risks for handling their contents (**Figure 1**).

- **Water**

Tap water is a mixture containing anions (chloride, carbonate, hydrogen carbonate, etc.), cations (calcium, magnesium, sodium, etc.), dissolved gases(carbon dioxide, oxygen, etc.), molecules ($SiOH_4$, ...), suspended matter (sand, ...), and sometimes bacteria.

- Demineralized or "permuted" water. Tap water is filtered and passed through ion exchange columns. At the outlet of the last column, this water is freed from cations and anions.
- Distilled water, obtained by distillation, is stripped of anything that is not volatile and isproduced sterile.
- Tap water that is allowed to seep under pressure through a membrane that lets almost no molecules larger than H or ions pass through O_2 is known as reverse osmosis water.
- Several methods can be combined to produce ultrapure water, containing lessthan one impurity per billion water molecules (except H+ and OH-).

- **Material**

a. Measuring devices

In addition to scales, there are specific chemistry devices. You will handle pH meters and voltmeters that measure electrical voltages in relation to the concentration of dissolved species. There are also conductivity meters, which measure the conductivityof a solution containing dissolved ions.

These devices use electrodes that are fragile (the slightest shock can be fatal to them) and very expensive. When they are not used for measurements, they must be immersed in purified water; to clean them, simply use a squeeze bottle of demineralizedwater and wipe them (without rubbing) with Joseph paper. Colorimeters will also be used, these are devices designed to measure the light absorption by a colored solution for a chosen wavelength, generally corresponding to the complementary color of the solution (**Figure 2**).

Balance **pH meter** **Voltmeter**

Figure 2. Measuring devices

a. Glassware, Containers

These are beakers, crystallizing dishes and Erlenmeyer flasks. They are all made of borosilicate glass with a low coefficient of expansion, which allows them to withstand thermalshocks; The graduations on these containers are for information

purposes only. They are not intended for precise measurement of volumes (**Figure 3**).

Figur 3. Glassware and containers

a. Glassware, measuring volumes

The graduated test tube : It is used to collect a variable but defined volume of any liquid. It is the utensil leading to the greatest uncertainty on the value of the measured volume (**Figure 4)**.

Figure 4. Graduated test tubes

The graduated flask : It is used to dilute a sample; the volume indicated is that contained in the vial, not that which can be extracted, because there will always besome left inside (**Figure 5**).

Figure 5. Graduated test tubes

*Use:

The sample is placed in the flask (a previously weighed solid, or a given volume of aliquid) then water is added up to half (approximately) of the flask.

Stopper and shake to obtain a homogeneous solution. Then add more water upto the mark engraved on the neck of the flask (**Figure 6**).

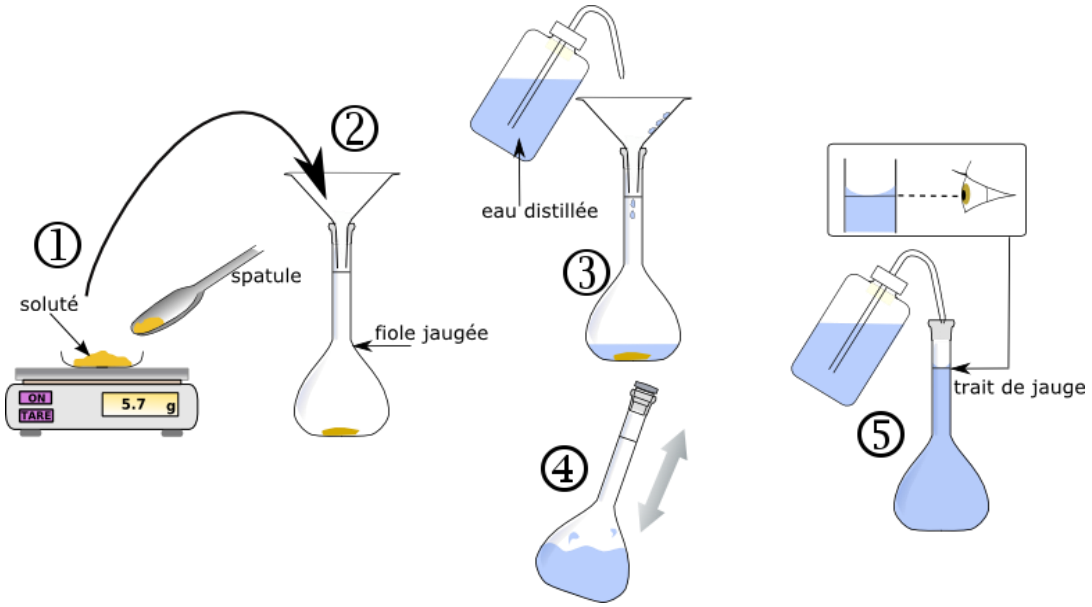

Figure 6: Preparation of a solution

- ✓ **Pipette** :

It is used to collect a precise volume, generally small, of a liquid. Thereare several types of pipettes: graduated pipettes whose volume is fixed as for graduated flasks and graduated pipettes whose volume is variable (**Figure 7**).

Figure 7: Preparation of a solution

**Use:*

The pipette should always be equipped with a Pro-pipette or a bulb. The pipette is filled above the upper mark with the liquid and then emptied until the base of the meniscus is tangentto the upper mark. The contents of the pipette are then emptied into the desired container by pressing its tip against its inclined wall.

-***The oil can :*** It is used for dosages. It is graduated and allows you to gradually addprecise volumes.

Use : Rinse the burette with a small volume of the filling solution. Start filling, leaving the tap open and with a container underneath, so as to expel the air bubblethat is often found in the tap or underneath. Close and fills above the upper line. The zero is then adjusted by bringing the lower part of themeniscus tangentially to the mark.

Tolerance standards (volumetric glassware):

	Volumetric pipettes			Graduated pipettes			burettes		Volumetric flasks		
Ability (ml)	5	10	20	2	5	10	25	50	50	100	250

Class A	-0.01	-0.015	-0.02	-0.01	-0.02	-0.04	-0.05	-0.05	-0.06	-0.1	-0.2
Class B	-0.015	-0.0225	-0.03	-0.015	-0.03	-0.06	-0.1	-0.1	-0.09	-0.15	-0.3

In all cases, care should be taken to avoid parallax errors by placing the object at the level of the meniscus of the aqueous solution.

- Other containers

There are also wash bottles used to deliver volumes of liquid (usually purified water) controlled by the experimenter's hand, and separating funnels (**Figure 8**).

Figure 8. Other containers

You should always pay attention to the contents of a wash bottle, which will often be water but could also be alcohol, acetone or ether. When the liquid is volatile (acetone and especially ether), it can create excess pressure when it evaporates and the liquid escapes spontaneously. The separating funnel (opposite) is used to separate two immiscible liquids of different densities (**Figure 9**).

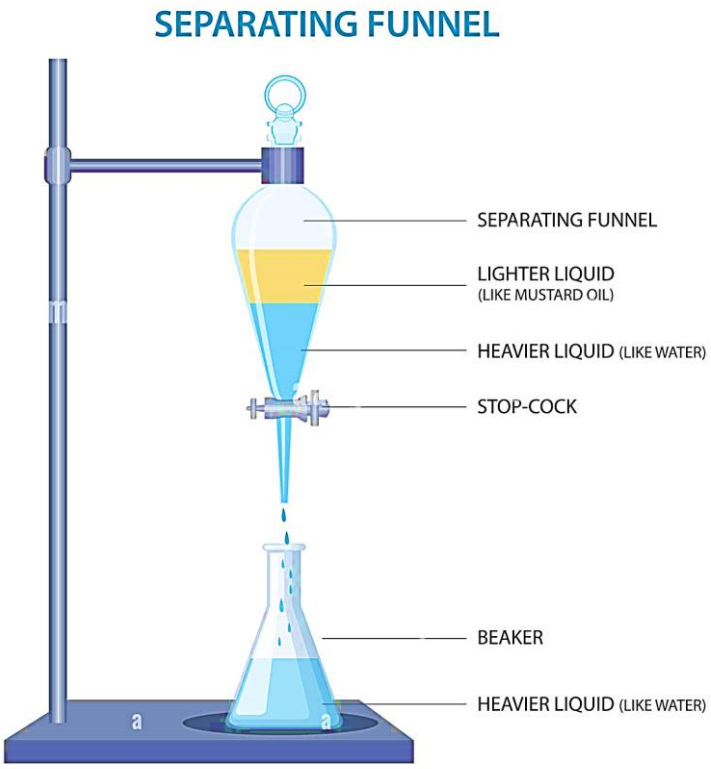

Figure 9. Separating funnel

2. IDENTIFICATION TECHNIQUE

2.1. Thin layer chromatography (TLC)

It is a technique that allows the separation of chemical species present in a homogeneous mixture, and therefore to control the purity of a sample. It also allows the identification of these chemical species contained in the sample. The samples to be tested, as well as the pure species **witnesses**, are arranged on a **chromatography plate (fixed phase: silica gel)** diving into an **eluent (mobile phase: solvent)**. Attached below isthe sequence of steps during the implementation of a CCM (**Figure 10**).

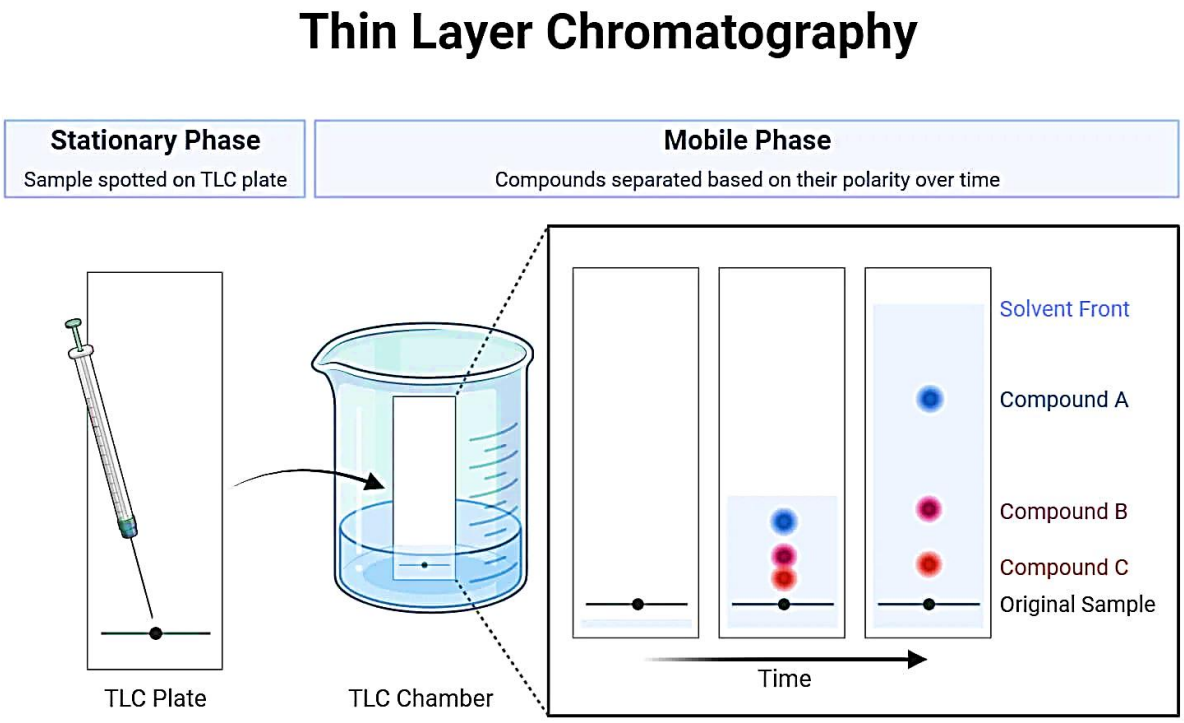

Figure 10. Thin Layer Chromatography

Practical work

PW N°1: Aspirin Synthesis

Introduction

Acetylsalicylic acid is the active ingredient of the *aspirin*. It comes in the form of a white solid with a melting point of **133°C**. It has analgesic, antipyretic,anti-inflammatory and anticoagulant properties (**Figure 11**).

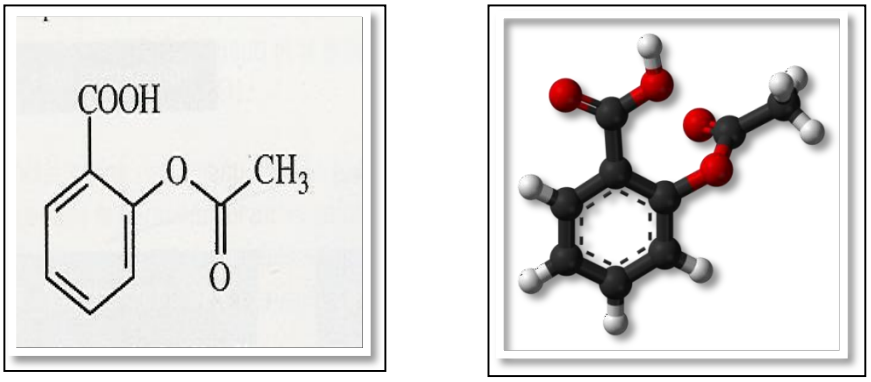

Figure 11. Acetylsalicylic acid

L'acetylsalicylic acid was originally synthesized by **Gerhardt** (1853), and is one ofthe first drugs of purely synthetic origin. Despite some undesirable side effects (ulcers, teratogenic effects, etc.), the **aspirin** remains one of the most widely used **analgesic products.**

To facilitate the solution of the **acetylsalicylic acid**, the titration is done in a water/ ethanol mixture (**1/1**). Therefore, the scale of **pH** is slightly offset and the **pH** from the half-equivalence point is located

Purpose: The goal of this lab is to synthesize aspirin, the oldest drug in the world.

Principle:

In 1830, Leroux isolates the active principle and names it salicilin. In 1860, *Kolbe* synthesizes salicylic acid from phenol and carbon dioxide. But this acid is bitter and poorly tolerated in the stomach. Aspirin or acetylsalicylic acid was manufactured and marketed for the first time in 1899 by theGerman company Bayer. Thanks to its antipyretic, analgesic and anti-inflammatory properties, it is one of the most consumed drugs in the world. The aspirin synthesis reaction has the equation (**Figure 12**):

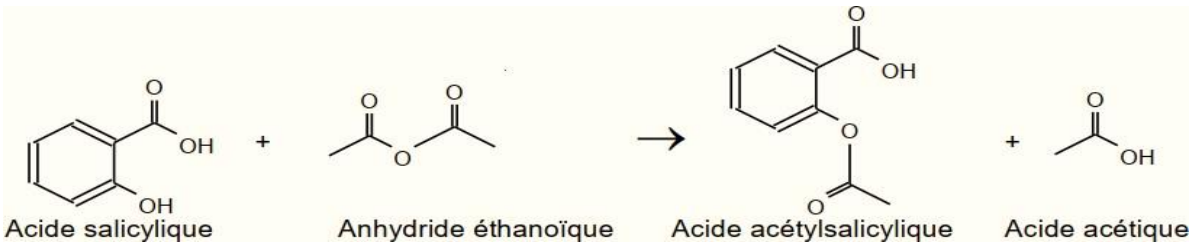

Figure 12. Aspirin synthesis

Materials and reagents: salicylic acid, acetic anhydride, sulfuric acid, ethanol, distilled water, pipetteand dropper, air condenser, stirrer + hot plate, 125 ml Erlenmeyer flask, 500 ml beaker, long spatula, Bûchner filter, methyl green.

Operating mode

1. Introduce 5.0 g of powdered salicylic acid into a round-bottomed flask.
2. Add 8.0 mL of acetic anhydride under the hood.
3. Add 5 drops of sulfuric acid then shake to dissolve the mixture and fit an air condenser onto the flask.
4. Heat the mixture in a water bath (60°C) for approximately 20 minutes while stirring regularly.
5. Remove the Erlenmeyer flask from the water bath while leaving the coolant in, wait for the mixture to cool.
6. Gradually introduce, in small quantities, approximately 70 mL of cold distilled water through the top of thecondenser.
7. Place the flask in an ice water bath for 10 minutes. Do not shake. We observe the crystallization aspirin.
8. Filter through Buchner assembly and rinse with cold distilled water.
9. Place the raw aspirin in a flask, add 10 ml of ethanol and 20 ml of hot water, heat directly on thehot plate (70°) and shake with a stirrer until redissolved.
10. Remove the balloon from the plate and leave to cool for a few minutes on the table, then place the balloon in a ice bath.
11. When recrystallization is complete, filter again through Buchner. The purified aspirin obtained.

12. Dry the purified aspirin crystals between two pieces of filter paper and weigh the aspirin obtained byplacing it in a previously weighed watch glass.
13. The aspirin obtained is impure. It is necessary to purify it. The method used is recrystallization.

Objective :

1. Carry out a step in the synthesis of aspirin
2. Purify the product obtained by recrystallization and identify it
3. Calculate the reaction yield

Questions :

1- Give the semi-structural formulas of salicylic acid, ethanoic anhydride and aspirin.

2- Write the balance equation of the reaction corresponding to the synthesis of acetylsalicylic acid

3- Why should we use the minimum amount of ethanol to dissolve acetylsalicylic acid

4- what is the principle of recrystallization

5- What is the effective yield of this synthesis (mass of product obtained is 2.5 g

PW N°2 : Extraction and identification of anethole

Introduction

Anethole is a type of orally active aromatic compound that is widely found in nature and used as a flavoring agent. Anethole possesses **anticancer**, **anti-inflammatory**, **antioxidant, antibacterial, antifungal, anesthetic, estrogenic, central nervous system depressant, hypnotic, insecticidal, and gastroprotective effects. Anethole** can be used in the study of oxidative stress-related skin diseases and prostate cancer

Objective: Anethole is extracted from star anise. Then we will identify the extracted substance (**Figure 13**)..

Figure 13. Natural anethole

General

Anethole gives its taste to anise, fennel and star anise. It is an unsaturated aromatic ether. Anethole appears as white crystals at room temperature. In liquid state, anethole is colorless and hydrophobic. Anethole has a distinctly sweet taste and is thirteen times sweeter than sugar. The taste felt is pleasant even at high concentrations. It is slightly toxic and can be irritating in large quantities.

- **Anethole**

- ✓ **Chemical Formula:** $C_{10}H_{12}O$
- ✓ **Molar mass:** 148.205 g/mol
- ✓ **Density:** 0.998 g/cm3
- ✓ **Melting point:** 20 to 21 °C (68 to 70 °F; 293 to 294 K)
- ✓ **Boiling point:** 234 °C (453 °F; 507 K); 81 °C (178 °F; 354 K) at 2 mmHg

Operating methods :

a. Extraction

1. Using a mortar, grind approximately exactly 1 g of star anise.
2. Weigh the powder obtained and put it in an Erlenmeyer flask containing 10 mL of dichloromethane or cyclohexane and cap.
3. Shake for approximately 30 minutes then filter. Rinse the filter with a little dichloromethane or cyclohexane.
4. The filtrate is transferred into a separatory funnel containing 10 mL of distilled water, shaken (degas!).
5. The recovered organic phase is dried over sodium sulfate, filtered and evaporated the solvent under reduced pressure (**Figure 14**).
6. Weigh the extract.

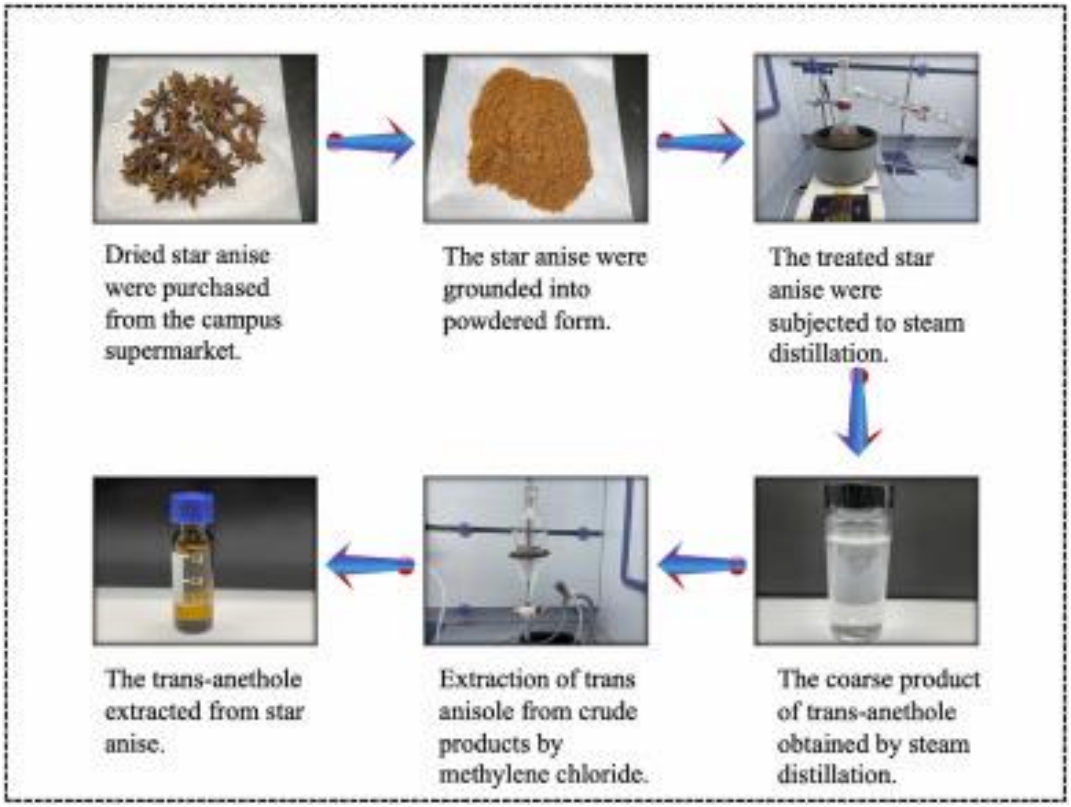

Figure 14. Extraction procedure

b. Identification : Carry out a TLC:

Eluent: Dichloromethane / ethanol 96:4

Questions :

1-Explain “anethole is hydrophobic”.

2-Why is the filtrate “mixed” with water?

3-The density of water is 1.0; that of cyclohexane is 0.78.

* Diagram the separating funnel and specify the position of the organic phase and that of the aqueous phase. Justify.

4-Explain the role of sodium sulfate.

5-Why do we evaporate the solvent under vacuum?

6- Calculate the anethole content of star anise.

7- Identification results.

PW N°3 : Identification of paracetamol in a medication by Thin layer chromatography

Introduction

The paracetamol, a chemical species well known for its analgesic properties, is anamide obtained by reaction between ethanoic anhydride and para-aminophenol. Its structural formula is:

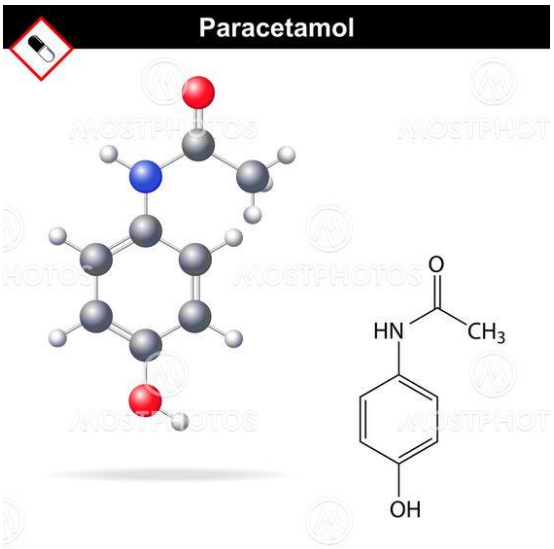

Figure 15. Paracetamol

Aim :

Identify the presence of the paracetamol in a medicine.

Method of operation:

1. Prepare the chromatography tank by pouring in the eluent, which is either diethyl ether or a mixture of chloroform and methanol in the proportions **60% - 40%**.
2. Prepare the solutions that will be studied by putting some crystals in **1 mL** solvent, which is the same mixture as the eluent.
3. Pure paracetamol
4. the powder obtained by crushing a tablet of Doliprane, Dafalgan and of Aspirin.

5. Place a drop of these solutions on the chromatography plate and dry.
6. Reveal the chromatogram under UV light and surround the spots.

Questions :

1. Give the principle of chromatography.
2. What can be said about the composition of these drugs?**?**
3. Then determine the frontal ratio of the highlighted constituents. Discuss the results obtained.

PW N°4 : Extraction and dosage of flavonoids and polyphenols from a medicinal plant

Biological material

Plant: scientific and vernacular name, date and region of harvest, identification (**Figure 16**).

Figure 16. ***Rosmarinus officinalis*** **L.**

Chemical materials

Methanol, distilled water, Folin-Ciocalteau reagent, $AlCl_3$, sodium carbonate, gallic acid.

Methods

Drying: in a ventilated place, away from light.

Grinding: using a mortar or electric grinder.

Maceration

- ✓ Weigh 10 g of the dry matter (powder) of a medicinal plant.
- ✓ Place the (10 g of dry matter) in an Erlenmeyer flask.
- ✓ Add 100 ml of ethanol to dissolve the dry matter.
- ✓ Shake the mixture then leave it for 24 hours.

Filtration: The mixture is then filtered through cotton and then filter paper using a funnel.

Drying: in the oven at 40°C.

Calculation of Yield: calculate the extraction yield per 100 g of dry matter

Determination of total polyphenol

We estimated the total phenolic content in the extract using the method of Folin-Ciocalteu's:

1. In brief, we mixed an aliquot of 100 µL of the stem extract with 500 µL of Folin-Ciocalteu's reagent for 4 min, followed by the addition of 400 µL of a 7.5% aqueous Na_2CO_3.
2. Then, absorbance of solution was determined at 765 nm after 2h of incubation. Polyphenolic content was expressed as µg gallic acid equivalent (GE)/mg dried extract (DE).
3. The amount of total polyphenols in different extracts was determined from a standard curve of gallic acid ranging from 0.00 to 160 µg/mL (**Figure 17**).

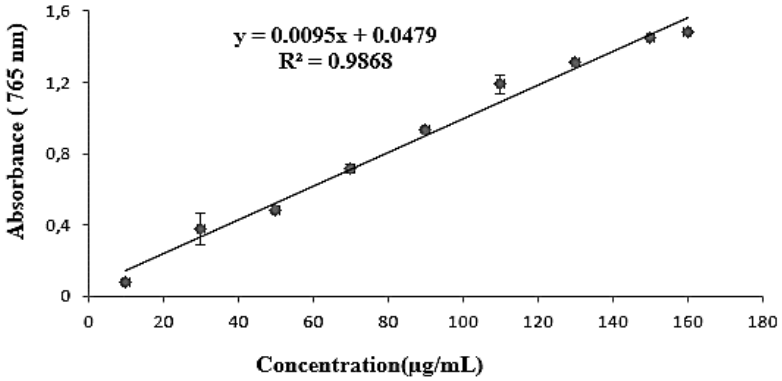

Figure 17. Standard curve of gallic acid. Each value represent mean ± SD (n = 3).

Determination of total flavonoids

The total flavonoids content was evaluated by the method of aluminum chloride.

1. According to this method, 1 mL of extract was added to 1 mL of aluminum chloride solution (2%).
2. This was followed by measuring the absorbance of the mixture at 430 nm versus a methanol blank, after 10 min of incubation.
3. Total flavonoids were reported as µg of quercetin equivalent (QE)/mg dried extract (DE).
4. The amount of total flavonoids in different extracts was determined from a standard curve of quercetine ranging from 0.00 to 40 µg/mL (**Figure 18**).

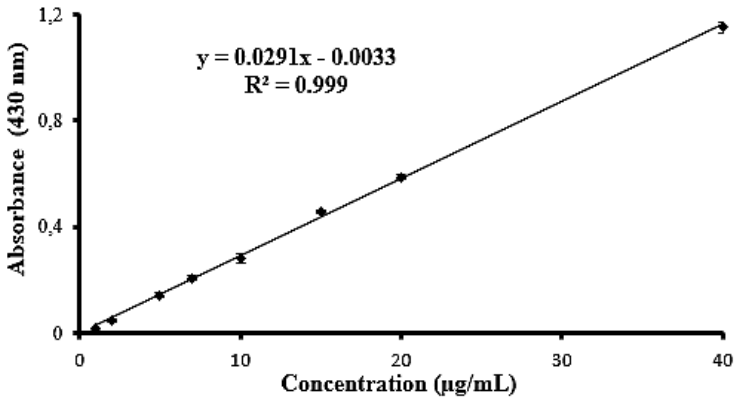

Figure 18. Standards curve of quercetine. Each value represent mean ± SD (n=3)

Questions :

1. Give the principle of extraction.
2. What can be said about the composition of these extracts
3. Cited other different extractions methods
4. Then determine the frontal ratio of the highlighted constituents. Discuss the results obtained.

PW N°5 : Antioxidant activity by DPPH radical scavenging activity

Introduction

Polyphenol antioxidant activity in extracts from medicinal plants depends on the structure of their functional groups. The multiple of hydroxyl groups are greatly influences several mechanisms of antioxidant activity such as scavenging radicals and metal ion chelation ability. Additionally, the position and the arrangement of the hydroxyl groups around the phenolic molecule is also important for anti-oxidative activity.

Purpose :

Determination of the antioxidant in different plant extracts (**Figure 19**)

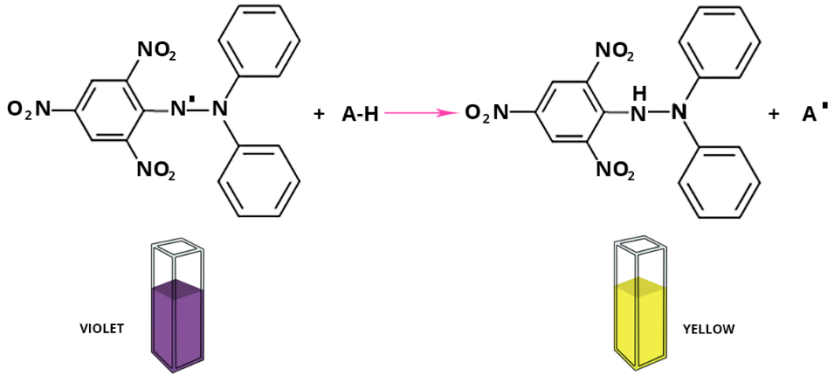

Figure 19. Antioxidant activity reaction

Methods

DPPH radical scavenging assay

Free radical scavenging capacity of the extracts was assessed using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) assay by measuring the decrease in the DPPH maximum absorbance at 517 nm.

1. In this method, 50 µL of various concentrations of the extracts/standard were mixed with 1.25 mL of DPPH solution in methanol (0.004%).

2. Absorbance of the sample was measured at 517 nm after a 30 min of incubation in the dark at room temperature.
3. Butylated hydroxytoluene (BHT) was used as positive control.
4. The scavenging capacity was calculated according to the following equation :

$$I\% = (A_{blank} - A_{test} / A_{blank}) \times 100.$$

Where A_{blank} = absorbance of the solution except the tested sample , and A_{test} = absorbance of the tested sample.

Questions :

1. Give the principle of the antioxidant activity.
2. What can be said about the percentage inhibition of these extracts
3. Cited other different antioxidant methods
4. Discuss the obtained results.

PW N°6 : *In vitro* cytotoxicity

Introduction

Toxicology is the study of the effect of chemical and biological substances on human health. Many sources exist that contain valuable information on toxicology testing methods comprising *in vivo* and *in vitro* testing methods. The study of the toxicity of a substance is the set of pharmacological tests, which determine the degree or the harmfulness of the latter in order to regulate its use. Natural antioxidant compounds like flavonoids and coumarins have been proposed as an effective approach for the prevention and treatment of multiple red blood cells (RBC) disorders ***via*** scavenging free radicals or lipid peroxyl inhibiting effect.

Method

In vitro cytotoxicity against red blood cells

Hemolysis test was performed using human blood of a healthy donor.

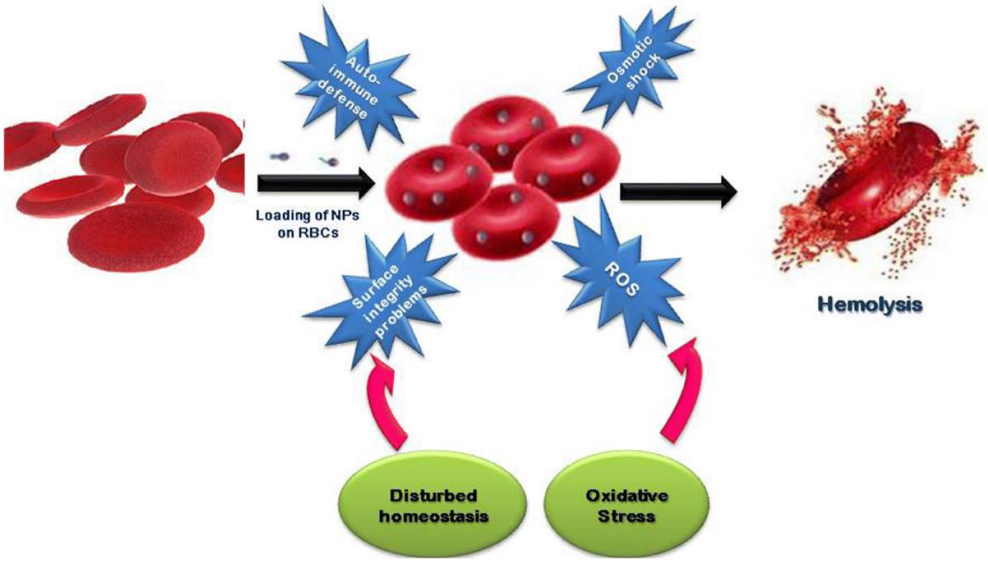

Figure 20. *In vitro* cytotoxicity against red blood cells

1. Whole blood was obtained from healthy young human donors and was collected according to the standard operating procedures.

2. According to this procedure, **human erythrocytes from blood were isolated by centrifugation at 3000 rpm for 10 min and washed** three to **four times with** NaCl (0.9%) solution until the supernatant became colorless.
3. After the centrifugation, the blood volume was measured and reconstituted as a 2% (*v*/*v*) suspension with isotonic buffer solution (10 mM sodium phosphate buffer pH 7.4).
4. Then, a mixture (1 mL) containing 500 µL of ButE/isolated compounds (0.5 and 1 mg/mL) or Triton X-100 (0.1%) and 500 µL of erythrocyte suspension (2%) was incubated at 37 °C for 1 h.
5. After the incubation periode, the suspension was centrifuged at 3000× *g* for 10 min. Absorbance of supernatant was determined by spectrophotometrically at 540 nm and hemolusis was calculated by the following formula:

$$\text{Hemolysis \%} = (\text{Abs}_{\text{test}} / \text{Abs}_{\text{Triton X-100}}) \times 100$$

where the Abs_{test} is the absorbance in the presence of tested compound, and $\text{Abs}_{\text{Triton X-100}}$ is the absorbance in the presence of Triton X-100 as a positive control. The positive control induces 100% hemolysis.

Questions :

1. Give the principle of ***in vitro*** cytotoxicity.
2. What can be said about the toxicity of the extracts used in this experience
3. Cited other different toxicity methods
4. Discuss the results obtained.

PW N°7 : *In vivo* acute oral toxicity

Introduction

Acute toxicity refers to those adverse effects occurring following oral or dermal administration of a single dose of a substance, or multiple doses given within 24 hours, or an inhalation exposure of 4 hours.

Experimental animals

Adult female albino mice (25–30 g) were used. These animals were purchased from 'Institut Pasteur d'Algérie', Algiers. Mice were housed in cages under standard conditions of 12:12 h light/dark cycle and 25 ± 1°C for seven days before the experiments. They were given free access to water and standard diet (*ad libitum*), and kept under standard conditions mentioned in the Animals By-Laws N°.

Acute oral toxicity study

The acute toxicity of the crude extracts of stems and roots is evaluated in rats, using the internationally accepted guidelines (**Figure 21**).

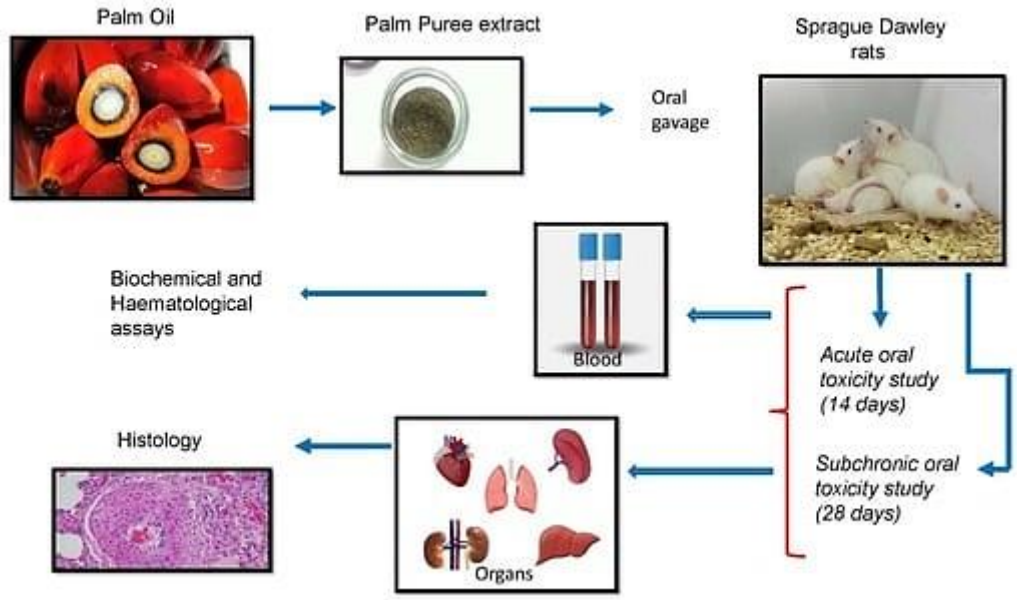

Figure 21. Acute oral toxicity

1. Mice were divided into three groups (n = 5) and subjected to fasting for 12 h before the experiment.
2. Doses of extract (2 and 5 g/kg in 1 mL H_2O, respectively) were orally given to each rat in the first and second groups, respectively, whereas rats in the third group (negative control) were given distilled water.
3. Body weight and observations of symptoms of toxicity were recorded systematically for 48 h and then once a day for 14 days.
4. Surviving animals were sacrificed then blood and organs were subjected to biochemical and histological analyses, respectively.

- ***Questions :***

    1. Give the purpose of acute oral toxicity.
    2. What can be said about the toxicity of this extract
    3. Discuss the results obtained.

PW N°8 : Dissection of mice

Protocol:

1- **Pin the animal ventral side up**, on the **clean dissection board** (Fig. 23A). Avoid holding the animal in one hand, so as to keep your hands clean and to properly cut the organs.

2- **Clean the ventrum** with cotton wool soaked with ethanol to avoid introducing hairs in the body and infecting organs (Fig. 23B).

3- Pinch and raise the skin with dissecting forceps (Fig. 23C).

4- Cut through the body wall muscles just anterior to the genital opening and continue just to one side of the midline on the ventral side until the thoracic cavity (Fig. 23D). Use blunt-end scissors (either two blunt-end scissors or one blunt, one sharp point scissors) to avoid damaging the organs.

5- Pull the skin to keep the fur away from the body on each side of the thoracic cavity (Fig. 23E).

6- Cut large parts of **lungs** and place them quickly in the cryogenic tubes (Fig. 23F). We recommend keeping some more parts of lungs in another tube filled with RNA later.

7- Clean forceps and scissors (bleach + water + ethanol).

8- Move the stomach to the left to find the **spleen** beneath (the spleen can be identified by its triangular shape).

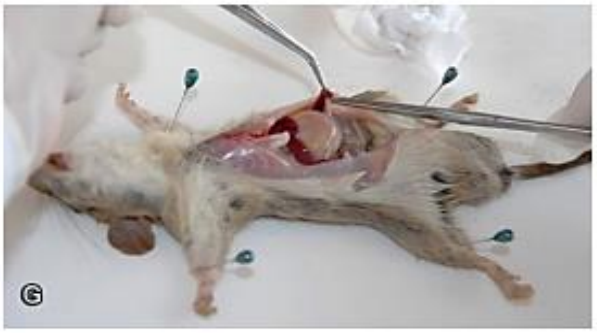

Figure 22. Part one of separation of organs

9- Pull the spleen with forceps and carefully remove the white tissue with sharp points scissors (Fig. 23G). Cut the whole spleen and place it on a filter paper to check its **weight** (in mg) with a precision balance (to get information for immuno-competence) (Fig. 24H). Then place it in the cryogenic tubes with and without RNA later.

10- Clean forceps and scissors (bleach + water + ethanol).

11- Move the intestines to find the kidneys beneath.

12- Separate the **kidneys** and place them entirely in two cryogenic tubes, one with and one without RNA later (if the kidneys are too big, cut it in pieces) (Fig. 24I).

13- Clean forceps and scissors (bleach + water + ethanol).

14- Cut the **diaphragm** and place it entirely in a cryogenic tube (Fig. 24J).

15- Place the **cryogenic tubes in the liquid nitrogen tank** (Only open the tank one time at the end of dissection of each animal to limit the evaporation of nitrogen).

16- Clean forceps and scissors (bleach + water + ethanol).

17- Cut a piece of **liver** and place it in an Eppendorf® tube prepared with absolute ethanol for DNA analysis (Fig. 24K).

18- Place the **Eppendorf® tubes in a fridge** or foam box with ice (if no fridge is available).

19- Clean forceps and scissors (bleach + water + ethanol).

20- Observe the **genital organs** to confirm the sex identification and eventually count embryos or measure the testes.

21- Cut the ears for studying Chigger mites (Fig. 24L) and place one in an Eppendorf® tube prepared with absolute ethanol for DNA analysis and the other one in a cryogenic tube for isolation of pathogens in Chigger mites.

22- Attach the identification tag to one leg. Use narrow point forceps to perforate the skin and introduce the string.

23- Verify that all measurements and samples were taken (Fig. 24M).

24- Place the animal into a watertight jar, filled with 70 degrees ethanol.

25- Clean the dissection board (bleach + water + ethanol).

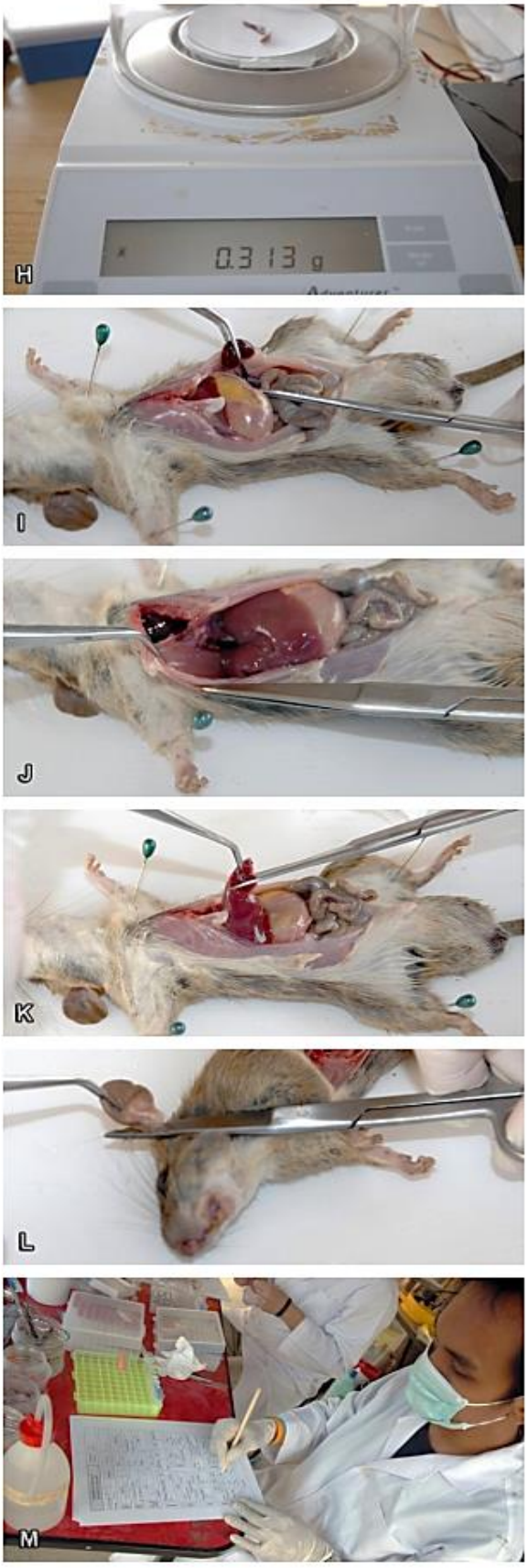

Figure 23. Part two of separation of organs